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Canadian Journal of Research, Section F (Technological Sciences)	Canadian Journal of Technology

In order to preserve continuity the present sequence of volume numbers will be retained, and in each case the volume for 1951 will be Volume 29.

The subscription rates for the Journals will remain as at present.

Canadian Journal of Research

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THE PROTEOLYTIC ENZYMES OF MICROORGANISMS

I. SURVEY OF FUNGI AND ACTINOMYCETES FOR PROTEASE PRODUCTION IN SUBMERGED CULTURE[1]

BY W. M. DION[2]

Abstract

Of 289 fungi and actinomycetes tested, only 20 were found to produce high yields of proteolytic enzymes when grown in submerged culture. Strains of *Gliocladium roseum* and *Alternaria tenuis* were found to be consistently proteolytic, and other good strains were also found in the genera *Chaetomium, Sordaria, Epicoccum, Rhizoctonia,* and *Streptomyces.*

Introduction

The fact that proteins are decomposed by the activities of microorganisms is so well known as to need no further introduction. However, the enzymes which bring about this decomposition are less well known, although the enzyme system of one or two organisms has been studied in detail. A considerable amount of work has been done on bacterial proteases, but the enzymes produced by molds and actinomycetes have received little attention. Mold proteases are important in the cheese industry where species of *Penicillium* are responsible for the ripening and flavors of Roquefort and Camembert cheese, and are used for chill proofing beer.

Among the molds and actinomycetes a few genera have been singled out for special studies and little is known about the proteolytic enzymes of the other genera. Thus, members of the aspergilli and penicillia have been studied in detail with occasional references to the proteolytic activities of *Fusarium* spp. and *Actinomyces griseus.*

Mold proteases have mostly been measured by the estimation of the extent of hydrolysis obtained when the organism was grown directly on a protein substrate, or by the determination of the activity of the enzyme present in the mycelium. Estimations of proteolytic power by measurement of the amount of amino and ammonia nitrogen produced during growth are subject to some obvious disadvantages. Waksman and Lomanitz (12) found that species of *Trichoderma* and *Zygorhynchus* would utilize proteins and amino acids as sources of both carbon and nitrogen. If a carbohydrate was present

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[2] *Mycologist.*

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in the substrate, the amino acids were utilized only as a source of nitrogen, and ammonia did not accumulate. When the amino acids were utilized for carbon as well as nitrogen, ammonia did accumulate. The behavior of the actinomycetes, however, was not affected by the presence of a carbohydrate source. These organisms split proteins and amino acids with the liberation of ammonia independently of the presence or absence of carbohydrate. Waksman and Lomanitz concluded (12) that the measurement of ammonia produced during growth was not a reliable index of proteolysis.

Jensen (6), making a study of the fungus flora of Danish soils, found that *Citromyces glaber*, *Fusarium orthoceras*, and *Actinomyces griseus* were the most proteolytic of a number of organisms tested, that is, produced most amino and ammonia nitrogen when grown on a protein substrate. Sukhenko and Semovskiki (9) found that when *Fusarium* sp. was grown on a gelatin medium the total nitrogen in the medium decreased, while amino and ammonia nitrogen increased. In two hours the fungus had hydrolyzed about 80% of the gelatin into smaller fragments, but only about 2% was decomposed by the breaking of peptide linkages.

Other methods of determining the proteolytic activity of microorganisms include the use of dried ground mycelium, and extracts of mycelium. Some of the most detailed work on mold proteases was done by Berger, Johnson, and Peterson (2) who investigated about 30 species of *Aspergillus* and *Penicillium*. With extracts of fresh mycelium they identified one proteinase and at least five peptidases. Thibodeau and Macy (10) used dried ground mycelial powder for studies on the proteolytic enzymes of *Penicillium roqueforti*. Ayres and Tobie (1) extracted bran cultures of over one hundred molds and determined their proteolytic activity and they found that the aspergilli produced the most active proteases of the molds tested. Jensen (6), working with soil fungi, found that members of the Mucoraceae, penicillia, and in some cases fusaria, were the most active protein decomposers.

The present work was undertaken in order to make a survey of molds and actinomycetes to determine their capacity to produce proteolytic enzymes when grown in submerged culture. By this method only the extracellular enzymes which are released into the culture medium are estimated.

Experimental

Methods

The cultures were carried on potato dextrose agar plates with the exception of a few isolates of *Streptomyces* which did not grow well on this medium and were cultured on Czapek-Dox agar. When the colonies had become large enough, a piece of approximately 4 sq. cm. was cut out and ground up in a Waring Blendor with about 20 ml. of sterile water. Two milliliters of this mycelial suspension was inoculated into the experimental flasks.

A medium containing 3% glucose, 1% Klim, 0.1% monobasic potassium phosphate, and traces of magnesium, iron, and zinc sulphates had been found to be suitable for protease production. This medium had a pH of 6.2 after

sterilization, but since a number of organisms produced organic acids during growth, 0.5% calcium carbonate was added to all the fungal cultures and 0.5% magnesium carbonate to the actinomycete cultures. The test cultures were grown in 500 ml. Erlenmeyer flasks containing 100 ml. of medium.

The majority of the fungi were incubated for four days at 30° C. on a Gump shaker rotating at 250 r.p.m. However, a number of very rapidly growing molds, such as *Neurospora*, and some of the Mucorales and aspergilli, were taken off after only two days, while all the *Streptomyces* isolates were incubated for five days.

After growth, the mycelium was filtered off, and when necessary the filtrate centrifuged to remove all small fragments and cells. It has been shown (see Paper II of this series) (4) that the activity of the proteases is considerably decreased by dilute acid, so that if the hydrogen ion concentration of the culture medium was greater than pH 5.5 after growth, the flask was discarded and the culture regrown with additional carbonate in the medium.

Tests for Proteases

The culture liquid was adjusted to between pH 6.3–6.7. Filter paper discs 12 mm. in diameter were dipped into the liquid and incubated on unbuffered 1% gelatin–agar plates for 24 hr. at room temperature, and the presence of proteolytic enzymes was indicated by the appearance of clear zones around the discs. (A phosphate buffer was found to prevent the formation of zones.) Two types of these zones were produced. The first tended to be rather faint and slightly opaque and even when very wide never indicated a really active enzyme. The second type was very clear and well defined, and often a narrow ring of this type resulted from a potent enzyme. The zones varied from 2–6 mm. in width but the size was not always a reliable indication of the amount of enzyme present as estimated by the formol titration. However, in no instance was any significant amount of enzyme found in a culture filtrate which failed to produce such a zone.

The amount of enzyme present in all cultures producing clear zones was then estimated by Sorensen's Formol Titration under the following conditions. Two milliliters of the culture solution were incubated with 10 ml. of a 1% gelatin solution buffered at pH 6.7 with $M/15$ phosphate buffer. Five-milliliter samples of the hydrolyzing mixture were used for the determination of amino groups by titration with 0.05 N sodium hydroxide with phenolphthalein as indicator. The titration difference after five hours' hydrolysis at 37° C. $\pm$ 0.1 was taken as a measure of proteolytic activity. Preliminary work showed that for accurate results gelatin should be titrated to pH 7.0 before the addition of formaldehyde, and then brought to pH 9.0. Since this was impractical when the end point was determined with phenolphthalein (at approximately pH 8.5), it was found to be more accurate to use the total titer rather than the titer after the addition of formaldehyde. For the small amounts of hydrolysis obtained in five hours' incubation, the total titer agreed fairly closely with

determinations on the same sample by the Van Slyke method for amino N and also with the Formol Titration using a glass electrode and the method described later (see Paper III of this series) (8). For this reason, where phenolphthalein has been used for determination of the end point, the results are expressed as increases in titer, and not as percentage hydrolysis.

The cultures were graded into the following groups. Those producing no clear zones, or giving a titration difference of less than 0.5 ml. of sodium hydroxide, were discarded as producing no significant amount of enzyme. Those giving a titration of 0.5–0.75 ml. were graded as low; between 0.75–1.00 ml. as moderate; and over 1 ml. of sodium hydroxide as good protease producing cultures.

It should be mentioned that when cultures are designated nonproteolytic, the term is not intended to mean that the organism does not produce any proteases, but that the enzymes are not released into the medium in appreciable quantities under submerged culture conditions. Probably all commonly occurring fungi and actinomycetes are able to decompose proteins when the mycelium is directly in contact with the substrate.

Notes on the Cultures

Most of the cultures tested were isolated from the soil. About 60 cultures were collected from other sources such as meat paste, cheese, moldy fruits and vegetables, and rust cultures. During an investigation by Dr. G. A. Ledingham into the effects of various substances on the germination of *Puccinia graminis* and *Melampsora lini*, the rust cultures were rapidly overgrown by fungi introduced with the rust spores, and the old cultures provided a convenient source for the isolation of a variety of fungi. Two cultures of *Coniophora puteana* and *Fomes pini* were received from Dr. C. G. Riley of the Dominion Laboratory of Forest Pathology, Saskatoon, and 14 cultures of *Alternaria* and *Stemphylium* from Dr. J. W. Groves, of the Department of Agriculture, Ottawa. Cultures of a number of fungi and actinomycetes were obtained from Prof. T. C. Vanterpool of the University of Saskatchewan.

It has not been possible in all cases to determine the species to which the fungi should be assigned. In most cases the genus has been determined, although 34 isolates, mostly sterile mycelial cultures, have not yet been identified. The eight isolates of *Trichoderma* all produce green fruiting areas, although they vary in odor and pigment production, and have slightly different conidial shapes. However, they have all been classified as *Trichoderma viride* Pers. ex Fries in accordance with the views of Bisby (3). Two rust colored fungi, which agreed with descriptions of *Agrostalagmus cinnabarinus* have been identified as *Verticillium lateritium* (Fr.) Rabh., as suggested by Valenta (11). A species of *Gliocladium*, which is probably *Gliocladium roseum*, was isolated frequently from both soil samples and rust cultures, and most of the isolates were high protease producers. The culture is pure white when grown on potato dextrose agar, but certain strains produce a bright yellow water soluble pigment which diffuses into the agar. The two isolates

of *Epicoccum*, both proteolytic cultures, agreed with the spore measurements given by *Epicoccum nigrum*. The mycelium of both strains was very highly pigmented.

Results and Discussion

The results of the survey are tabulated in Table I. Twenty out of 289, or about 7% of the cultures tested, were good protease producers. In Table II these cultures are arranged in order of potency.

TABLE I

PROTEOLYTIC ACTIVITY OF A NUMBER OF FUNGI AND ACTINOMYCETES

(Figures represent the number of isolates tested)

Culture	Protease production			
	None	Low	Moderate	Good
Actinomycetes				
Streptomyces	29	11	6	3
Phycomycetes				
Peronosporales				
Pythium ultimum	1	—	—	—
Mucorales				
Mucor	9	—	—	—
Cunninghamella	2	—	—	—
Mortierella elasson	1	—	1	—
Rhizopus	3	—	—	—
Ascomycetes				
Sphaeriales				
Neurospora sitophila	—	1	—	—
Chaetomium	1	—	—	1
Sordaria	—	1	—	1
Endomycetales				
Ashbya gossipyi	1	—	—	—
Hypocreales				
Claviceps purpurea	1	—	—	—
Basidiomycetes				
Ustilaginales				
Ustilago zeae	1	—	—	—
Agaricales				
Coprinus	1	—	—	—
Coniophora puteana	1	—	—	—
Fomes pini	1	—	—	—
Polyporus anceps	—	1	—	—

TABLE I—*Continued*

PROTEOLYTIC ACTIVITY OF A NUMBER OF FUNGI AND ACTINOMYCETES—*Continued*

(Figures represent the number of isolates tested)

Culture	Protease production			
	None	Low	Moderate	Good
Fungi Imperfecti				
Sphaeropsidales				
Macrophomina phaseoli	—	—	1	—
Phoma	3	—	—	—
Sphaeronaema	1	—	—	—
Unidentified	4	—	—	—
Moniliales				
1. Pseudosaccharomycetaceae				
Wild yeasts	3	—	—	—
2. Sporobolomycetaceae				
Sporobolomyces	1	—	—	—
3. Moniliaceae				
Aspergillus niger	3	—	1	—
A. flavus-oryzae group	8	1	—	—
A. fumigatus group	5	1	—	—
A. candidus	—	—	1	—
A. glaucus group	2	—	—	—
Aspergillus sp.	2	1	—	—
Botrytis	1*	—	—	—
Candida lipolytica	1	—	—	—
Cephalosporium	1	3	1	—
Gliocladium roseum	—	—	1	5
Oospora lactis	2	—	—	—
Penicillium	22	7	—	—
Paecilomyces varioti	3	—	—	—
Scopulariopsis	4	—	—	—
Torulopsis	2	—	—	—
Trichoderma viride	4	—	3	1
Trichothecium roseum	2	1	—	—
Verticillium lateritium	2*	—	—	—
4. Dematiaceae				
Alternaria brassicicola	1	—	—	—
A. Dauci	1	—	—	—
A. dianthicola	—	1	—	—
A. linicola	1	—	—	—
A. radicina	1	—	—	—
A. Raphani	1	—	—	—
A. solani	1	—	—	—
A. tenuis	—	—	1	4
A. tenuissima	—	—	—	1
A. zinniae	1	—	—	—
Alternaria sp.	7	4	2	—
Cladosporium	3*	—	—	—
Coniosporium	2	—	—	—
Helminthosporium sativum	1	—	—	—
Helminthosporium sp.	2	—	—	—
Monotospora	2	—	—	—

* *Cultures grown at 25° C.*

TABLE I—*Concluded*

PROTEOLYTIC ACTIVITY OF A NUMBER OF FUNGI AND ACTINOMYCETES—*Concluded*
(Figures represent the number of isolates tested)—*Concluded*

Culture	Protease production			
	None	Low	Moderate	Good
Fungi Imperfecti—Concluded				
Moniliales (concluded)				
4. Dematiaceae (concluded)				
Periconia circinata	1	—	—	—
Stemphylium botryosum	—	—	1	—
S. consortiale	1	—	—	—
S. sarcinaeforme	1	—	—	—
Stemphylium sp.	2	1	—	—
Stachybotrys atra	1	—	1	—
5. Tuberculariaceae				
Epicoccum	—	—	1*	1*
Fusarium	12	7	2	1
Mycelia Sterilia				
Rhizoctonia solani	1*	—	—	1
R. crocorum	—	1	—	—
Unidentified				
	21	6	6	1
Total	192	48	29	20

* *Cultures grown at 25° C.*

The proteolytic cultures are representative of widely different groups of fungi and members of the same genus, and even species, vary from nonproteolytic types to high protease producers. This is comparable with the results of Le Mense *et al.* (7) who found that strains of the same species of *Aspergillus* varied tremendously in their ability to produce amylase in submerged culture.

It is noticeable that none of the aspergilli or penicillia produced appreciable amounts of proteases when grown in submerged culture. Members of both groups, and also of *Scopulariopsis*, are known to be so active in the breakdown of proteins that methods of extracting the proteases from the mycelium have been patented. It is apparent that the various methods of estimating the proteolytic power of an organism do not give comparable results. When grown in submerged culture the Mucorales, with the single exception of one species of *Mortierella*, were conspicuous for the complete absence of any detectable

TABLE II

PROTEOLYTIC CULTURES, LISTED IN ORDER OF THEIR ACTIVITY

PRL No.	Culture	Isolated from:	Titer (ml. 0.05 *N* NaOH)
86	*Gliocladium roseum*	Rust culture	2.01
79	*Gliocladium roseum*	Rust culture	1.88
498	*Epicoccum*	Rust culture	1.33
517	*Verticillium terrestre*	Soil	1.28
471	*Alternaria tenuis*	Orange	1.26
376	*Streptomyces*	Soil	1.24
369	*Alternaria tenuis*	Meat paste	1.23
232	*Fusarium*	—	1.19
501	*Gliocladium roseum*	Rust culture	1.17
439	*Alternaria tenuissima*	Contaminant	1.17
419	*Rhizoctonia solani*	Flax	1.14
301	*Alternaria tenuis*	Rust culture	1.07
131	*Gliocladium roseum*	Rust culture	1.07
257	*Sordaria*	Rust culture	1.05
319	*Chaetomium*	Contaminant	1.04
472	*Alternaria tenuis*	Orange	1.04
387	*Streptomyces*	Soil	1.02
397	*Streptomyces*	Soil	1.01
476	(Unidentified)	Rust culture	1.00
92	*Trichoderma viride*	Contaminant	1.00

proteases in the culture medium, and the same was true of 29 isolates of *Penicillium*. Of 25 isolates of *Aspergillus* only two were moderate protease producers.

A high proportion of moderate to good protease producing cultures was found in the genus *Alternaria*. These molds are very commonly found on decaying materials and may play an important part in the breakdown of proteins, although members of this genus have not previously been mentioned in work on the proteolytic activity of molds. From the few isolates tested of *Chaetomium* and *Sordaria*, it appears that these genera would be worth further investigation.

All six isolates of *Gliocladium roseum* proved to be very good protease producers in submerged culture, and PRL 79 and PRL 86 gave consistently higher protease yields than any other culture.

Almost half of the 49 isolates of *Streptomyces* showed some proteolytic activity. These organisms have long been known to be very active in the decomposition of proteins in the soil.

It may be concluded from this preliminary survey that no one genus is especially adapted to the breakdown of proteins. The proteolytic cultures were distributed in an almost haphazard way throughout the various genera investigated. Quite often the cultures isolated from proteinaceous substrates, such as cheese, did not produce proteases when grown in submerged culture, whereas those collected from other sources gave high yields of proteolytic enzymes.

It is possible that certain species may be characterized by their ability to produce proteolytic enzymes. Grimes, O'Connor, and Cummins (5) considered that the rate of liquefaction of gelatin brought about by certain species of *Phoma* was a criterion of sufficient importance to be used in the identification of the species. It can be seen in Table II that all the proteolytic *Gliocladium* cultures belonged to the same species, and that four out of the five protease producing cultures of *Alternaria* were strains of *Alternaria tenuis*.

The conditions under which this survey was conducted were necessarily standardized, and were not equally favorable for all the organisms tested, since it was impossible to determine the individual requirements of almost three hundred fungi and actinomycetes. The factors affecting the production of proteolytic enzymes in submerged culture by a few representative cultures are described in the second paper of this series.

Acknowledgments

The author is indebted to Prof. T. C. Vanterpool and Dr. C. G. Riley for the gifts of cultures. Grateful acknowledgment is made to Dr. J. W. Groves, not only for the cultures received, but also for the identification of the proteolytic cultures of *Alternaria*.

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THE PROTEOLYTIC ENZYMES OF MICROORGANISMS

II. FACTORS AFFECTING THE PRODUCTION OF PROTEASES IN SUBMERGED CULTURE[1]

By W. M. Dion[2]

Abstract

The main factors that influence the production of proteolytic enzymes by a few selected cultures have been studied. The time taken to reach the maximum yield of proteases is dependent upon the growth rate of each organism, and varies from two to five days. The fungi tested require the presence of an easily available carbohydrate source in addition to a protein substrate in order to produce high yields of proteolytic enzymes. The *Streptomyces* cultures will produce proteases in the absence of a carbohydrate source, but yields are generally low. The fungi studied will not produce significant amounts of proteases when grown on predominately inorganic nitrogen sources in contrast with the *Streptomyces* cultures, one of which produced almost as high yields of proteolytic enzymes when grown with sodium nitrate as when grown with Klim. Of a number of protein sources Klim and malt sprouts provided the best media for protease production. The temperature of incubation and pH of the culture medium are also important factors affecting the yield of proteolytic enzymes.

Introduction

A survey of fungi and actinomycetes (3) showed that a number of cultures were able to produce high yields of proteolytic enzymes when grown in submerged shake culture. Several of these cultures have been selected for a more detailed study of the factors that affect the production of proteases, and also for a study of the proteolytic enzymes themselves.

Only one member of the Phycomycetes, *Mortierella* PRL 26, was found that produced a measurable amount of proteolytic enzymes, and although this culture was not included in the category of good protease producing cultures it was selected as representative of the lower fungi. Two strains of *Gliocladium roseum*, PRL 79 and PRL 86, and one strain of *Alternaria tenuis*, PRL 369, were chosen from the Fungi Imperfecti, and two *Streptomyces* isolates, PRL 318 and PRL 376, from the Actinomycetes. Although the behavior of some other fungi has been investigated, most of the work reported in this paper has been done with these six cultures.

Experimental

The formol titration as outlined in the previous paper (3) was used for the estimation of proteolytic activity and the difference in titer after five hours' hydrolysis at 37° C. ± 0.1 taken as the measure of enzyme activity. With this method the rate of production of free amino groups during the hydrolysis of a protein is estimated, and thus no indication of the relative activities of

[1] *Manuscript received July 25, 1950.*

Contribution from the National Research Council, Prairie Regional Laboratory, Saskatoon, Sask. Issued as Paper No. 93 on the Industrial Utilization of Wastes and Surpluses and as N.R.C. No. 2232.

[2] *Mycologist.*

the various specific enzymes is obtained. When one culture is said to be more proteolytic than another, it is not known whether the increased activity is the result of a greater production of proteinases, or peptidases, or both. Conditions which are said to favor the production of proteases in submerged culture are therefore those most favorable to the whole proteolytic system, but not necessarily so for any one participating enzyme.

All figures given in this paper are averages of at least duplicate determinations; sometimes more replicates were used. In general, duplication of data was very good as the results were reproducible with each new batch of medium and fresh inoculum. With the exception of the *Streptomyces* isolate PRL 318, which steadily lost its activity, the growth and enzyme production of all the cultures used in this work were very consistent. Unless otherwise stated the cultures were grown on a medium containing 3% glucose, 1% Klim, 0.1% monobasic potassium phosphate, and traces of zinc, iron, and magnesium sulphates; 0.5% calcium carbonate was added to the fungal cultures, and 0.5% magnesium carbonate to the actinomycetes. One hundred milliliters of medium was contained in each 500 ml. Erlenmeyer flask, and after inoculation was incubated on a Gump shaker rotating at 250 r.p.m. Most fungal cultures were incubated for four days, *Alternaria* PRL 369 for three, and all *Streptomyces* isolates for four to five days. After growth the culture medium was freed from cellular material and tested for proteolytic activity.

Results

The following factors are the ones which have an important influence on the production of proteolytic enzymes in submerged culture.

Reaction of Medium

Since many enzymes are very sensitive to changes of pH, it was important to determine the reaction at which fungal and actinomycete proteases were most stable when held at 30° C., as this was the temperature at which the cultures were incubated.

Aliquots of the culture filtrates of *Gliocladium* PRL 79, *Mortierella* PRL 26, and *Streptomyces* PRL 376 were adjusted to hydrogen ion concentrations ranging from pH 3.5–8.0 with 1 *N* hydrochloric acid or 1 *N* sodium hydroxide. After incubation for 17 hr. at 30° C. the solutions were readjusted to pH 6.7, and their activity determined by the formol titration. As shown in Fig. 1 the proteases are most stable in neutral solutions about pH 6, although the enzymes from *Mortierella* PRL 26 did not appear to undergo any inactivation when held in alkaline solutions. It was therefore desirable to maintain the pH of the media between 5.5 and 6.5 during the growth of the fungal cultures. However, the Actinomycetes grew better and produced a higher yield of enzymes in more alkaline media. The reaction of the Klim–glucose medium used for screening was about pH 6.2 after sterilization and for nonacid producing molds needed no further adjustment. However, with molds producing

organic acids during the dissimilation of carbohydrates it was necessary to add buffers to prevent the pH from dropping. Proteases were never detected when the culture medium of any organism had been allowed to become

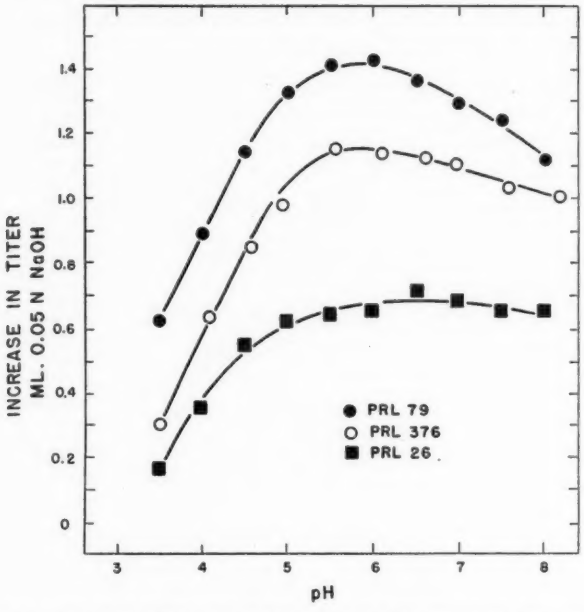

FIG. 1. *The effect of pH on the stability of mold and actinomycete proteases. Gliocladium PRL 79, Streptomyces PRL 376, and Mortierella PRL 26.*

as acid as pH 4.0. Table I gives the results of an experiment growing three different cultures with three different carbonates to adjust the pH of the medium. The *Streptomyces* grew best in an alkaline medium, as was expected, and produced its maximum protease when grown with magnesium carbonate. Barium carbonate or magnesium carbonate appeared to be most favorable for the *Aspergillus*, whereas calcium carbonate or barium carbonate was equally suited for growth of *Gliocladium* PRL 79. Magnesium carbonate depressed the protease yield in the latter.

Incubation Time

Proteolytic enzymes accumulate in the culture medium under favorable conditions during the period of active growth. In all the fungi tested the enzyme concentration reached a maximum after which it fell off, the rate of inactivation depending upon such factors as pH and amount of nutrients present. The rate of enzyme production appears to be correlated with the rate of mycelial production up to a certain point, after which any further increases in mycelial weight do not result in increases in proteolytic enzymes.

TABLE I

EFFECT OF CARBONATES ON PROTEASE PRODUCTION

Carbonate		*Streptomyces* PRL 318		*Aspergillus* PRL 336		*Gliocladium* PRL 79	
		pH after growth	Ml. 0.05*N** NaOH	pH after growth	Ml. 0.05*N** NaOH	pH after growth	Ml. 0.05*N** NaOH
None		5.76	0.32	4.45	0.47	6.10	1.25
$CaCO_3$	0.25%	5.91	0.37	—	—	6.20	1.34
	0.50%	6.10	0.40	—	—	6.20	1.39
	1.0%	—	—	5.41	0.65	—	—
$MgCO_3$	0.25%	7.40	0.80	—	—	7.00	0.94
	0.50%	7.90	0.76	—	—	7.30	0.60
	1.0%	—	—	5.15	0.89	—	—
$BaCO_3$	0.25%	6.90	0.18	—	—	6.70	1.40
	0.50%	6.74	0.20	—	—	6.70	1.44
	1.0%	—	—	5.94	0.76	—	—

* *Increase in titer.*

Those molds which produced abundant mycelium very rapidly, such as *Neurospora* PRL 482 and *Aspergillus niger* PRL 326, produced their maximum protease in two days, after which the concentration of enzyme dropped. Most fungi produced their maximum amounts of proteases in about three to four days. The *Streptomyces* isolates were slower growing than the fungi and generally reached their maximum protease production in about five days. In contrast with the mold proteases, the *Streptomyces* enzymes appeared to be stable for at least nine days and did not undergo the progressive inactivation characteristic of fungal proteases after reaching their maximum concentration in the medium. Some typical curves for protease production are given in Fig. 2.

Effect of Nitrogen Source

It has been shown by Waksman (7) and Tazawa *et al.* (6) that although species of *Penicillium* and *Aspergillus* will produce proteolytic enzymes when grown in still culture on a purely synthetic medium, they produce a much greater yield when grown on a protein substrate. As shown in Table II neither of the fungi tested would produce proteases when grown in submerged culture on an inorganic salts – glucose medium containing a small amount of yeast extract, although in several instances growth was good. *Alternaria* PRL 369 grew well on a purely synthetic medium, but *Gliocladium* PRL 79 required the presence of some growth substances. However, the addition of small amounts of organic nitrogen in the form of yeast extract did not result in the production of significant amounts of proteolytic enzymes. *Gliocladium* PRL 79 was grown on media in which the total amount of nitrogen was

constant, but consisted of varying amounts of protein nitrogen and inorganic nitrogen. It was found that the production of proteases began to drop as soon as inorganic nitrogen was introduced into the medium, and when half of

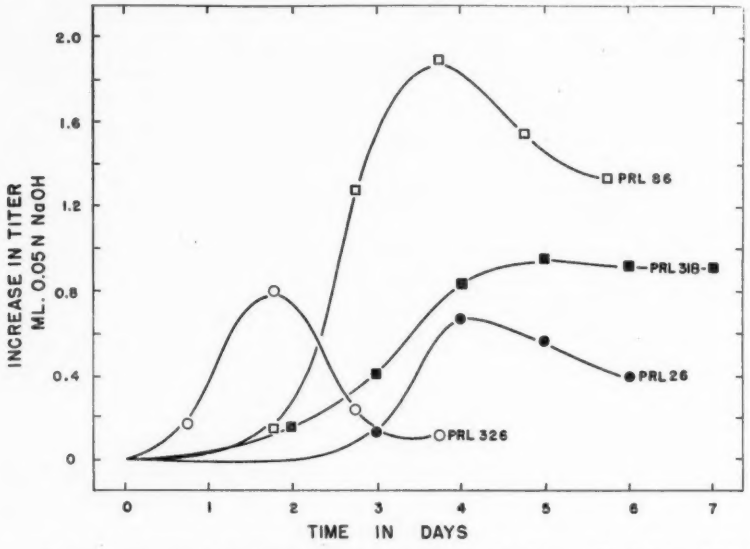

FIG. 2. *The rate of protease production in submerged culture. Glíocladium PRL 86, Streptomyces PRL 318, Mortierella PRL 26, and Aspergillus niger PRL 326.*

TABLE II

EFFECT OF NITROGEN SOURCE ON PROTEASE PRODUCTION

Nitrogen source*	Increase in titer—ml. 0.05 *N* NaOH			
	Glíocladium PRL 79	*Alternaria* PRL 369	*Streptomyces* PRL 318	*Streptomyces* PRL 376
Klim 1%	1.21	1.32	0.86	0.95
Malt sprouts 1%	1.51	1.10	0.16**	1.14
Casein 1%	0.46	0.85	—	0.66
Peptone 1%	0.45	0.56	0.62	0.78
Haemoglobin 1%	0.91	1.09	—	0.29
Gluten 1%	0.40	0.47	0.19**	0.30
Gelatin 1%	0.12	0.40	0.25	0.55
Egg white 1%	0.25	0.29	—	0.03
Asparagin 0.5%	0.15	0.14	0.26**	0.50
$(NH_4)_2SO_4$ 0.2%	0.17	0.18	0.20**	0.12**
$NaNO_3$ 0.2%	0.17**	0.12**	0.73	0.31
Urea 0.2%	0 **	0.15**	0.17**	0.15**

* *The medium contained 3% glucose, mineral salts, 0.5% carbonate, and 0.05% yeast extract in addition to the nitrogen source.*

** *Growth poor.*

the nitrogen was present in the inorganic form, proteolytic enzymes were hardly detectable. In contrast, however, both the *Streptomyces* isolates were able to produce proteases when grown with predominantly inorganic nitrogen sources, PRL 318 producing almost as high a yield of proteolytic enzymes when grown with sodium nitrate as when grown on Klim.

The protein used as a substrate has a considerable effect on the yield of proteolytic enzymes (see Table II). Of a number of proteins tested Klim and malt sprouts were found to be the most effective, perhaps partly because both constitute excellent growth media. Growth on haemoglobin by both fungi and actinomycetes was very heavy and rapid, but after four days' incubation the protease yield was moderate to low. It was found, on further investigation, that *Gliocladium* PRL 79 produced its maximum concentration of proteolytic enzymes in three days when grown on haemoglobin and that by the fourth day the concentration was already beginning to fall. However, the maximum reached was not as high as that on Klim by the fourth day. Protease yields on peptone and casein media were generally low, although growth was good, while gluten, gelatin, and egg white were not suitable substrates.

Effect of Carbohydrates Source

Provided that the carbohydrate was one that was easily available it had no effect on protease production. As shown in Table III *Gliocladium* PRL 79

TABLE III

THE EFFECT OF CARBOHYDRATE SOURCE ON THE PRODUCTION OF PROTEOLYTIC ENZYMES

Carbohydrate*	Increase in titer—ml. 0.05 *N* NaOH	
	Gliocladium PRL 79	*Streptomyces* PRL 318
None	0.04**	0.46
Glucose	1.25	0.76
Fructose	1.60	0.44
Lactose	0.40**	0.58
Mannose	1.28	0.74
Maltose	0.94**	0.60
Sucrose	1.68	0.55
Cerelose	1.72	0.74
Molasses	0.52**	0.85
Inulin	1.38	0.59
Dextrin	1.12	0.70

* *Medium consisted of 1% Klim, 3% carbohydrate, with minerals and 0.5% carbonate.*
** *Growth poor.*

grew readily on glucose, fructose, mannose, sucrose, cerelose, inulin, and dextrin. It grew very poorly, giving a low yield of proteases, on lactose, maltose, and molasses. The poor growth on molasses was probably caused by the excessive alkalinity of the medium which inhibited growth. *Streptomyces*

PRL 318 grew well on all the above mentioned carbohydrates including lactose, maltose, and molasses. The protease yield was low on fructose although growth was good.

When no carbohydrate was present in the medium, the culture liquid became highly alkaline and growth was inhibited. The fungi tested failed to produce any significant amounts of proteases when grown in submerged culture in the absence of a carbohydrate source but the actinomycetes tolerated the more alkaline conditions and proteolytic enzymes were produced; however, yields were generally low.

The Effect of the Amount of Protein and Carbohydrate Present in the Medium

High protease yields are always associated with good growth of mycelium and when growth is poor yields are low. If growth could be made to continue after the maximum concentration of proteolytic enzymes has been reached, the proteases should remain at their maximum level, or even increase.

Table IV shows the effect of adding fresh nutrients to cultures of *Gliocladium* PRL 79 after four days of growth, when the proteases had reached their greatest concentration and about 33% of the carbohydrate had been fermented.

TABLE IV

EFFECT OF ADDITIONAL NUTRIENTS ON PROTEASE PRODUCTION BY *Gliocladium* PRL 79

Days of growth	Control		Additional nutrients added*	
	pH	Increase in titer, ml. 0.05 *N*	pH	Increase in titer, ml. 0.05 *N*
4	6.33	1.78	6.19	1.54**
5	6.40	1.26	6.23	1.78
6	6.53	1.18	6.50	1.81
7	6.46	1.15	6.43	1.51
9	7.02	1.08	6.55	1.26

** Three grams glucose plus 1 gm. Klim in 30 ml. water added to experimental flasks on 4th day, controls left untouched.*

*** Drop in protease due to dilution factor after addition of nutrients as both sets of flasks had identical protease content when sampled previously.*

The presence of the additional nutrients delayed the inactivation of the enzymes, but the continued growth, although it restored the concentration of proteases present before the nutrients were added, did not lead to an increase in the concentration over the controls.

Fig. 3 shows the effect of increasing the amount of glucose on protease production by *Gliocladium* PRL 79. The medium contained 1% Klim and the yield of protease increased with increasing amounts of glucose until a maximum was reached at 8% glucose; 10% glucose resulted in a slight inhibition of protease production. When 2% or 3% glucose is present in the medium the protease yield begins to fall off after the fourth day, as has already been

reported. However, when more glucose was added the production of proteolytic enzymes increased until the sixth day at least, indicating that the enzymes are produced continuously as long as growth is possible.

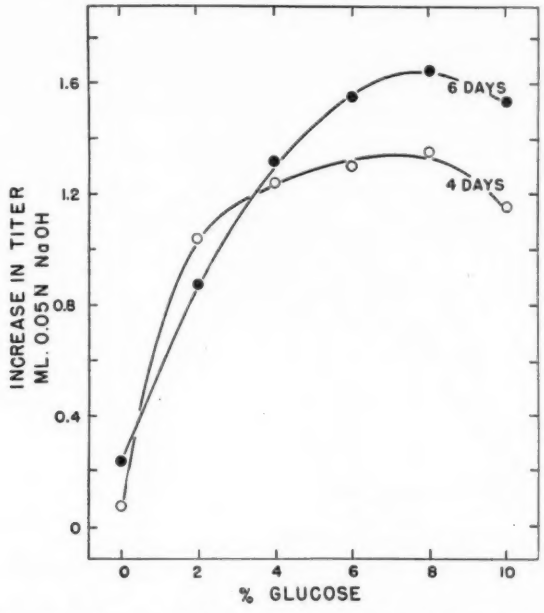

FIG. 3. *Effect of amount of glucose on the production of proteases by Gliocladium PRL 79.*

The effect of increasing the amount of Klim on protease production is shown in Fig. 4. *Gliocladium* PRL 79 was grown on a medium containing 3% glucose and varying amounts of Klim. Concentrations of Klim up to 2% resulted in increased protease yield, but concentrations of 3% and over caused a marked inhibition of protease production. Most cultures were very sensitive to the concentration of Klim in the medium. *Gliocladium* PRL 79 and *Streptomyces* PRL 376 produced higher yields of proteolytic enzymes when grown with 2% Klim than with 1% Klim. The reverse was true for *Alternaria* PRL 369 and *Streptomyces* PRL 318 and *Sordaria* PRL 257 which produced considerably less proteases when grown with 2% Klim. Whether the inhibitory effect is caused by a decrease in growth, or by the presence of an enzyme inhibitor in the Klim has not been determined. This decrease in enzyme yield is not characteristic of all protein sources. Table V shows the effect of increasing the concentration of malt sprouts in the medium of *Gliocladium* PRL 79. The quantity of malt sprouts appeared to have little effect on protease production, whereas the effect of glucose concentration was very marked.

When the optimum amount of Klim was used cultures of *Gliocladium* PRL 79 did not respond to variation in the amount of glucose in the medium to the same extent as when 1% Klim was used. Amounts of glucose varying from 3%

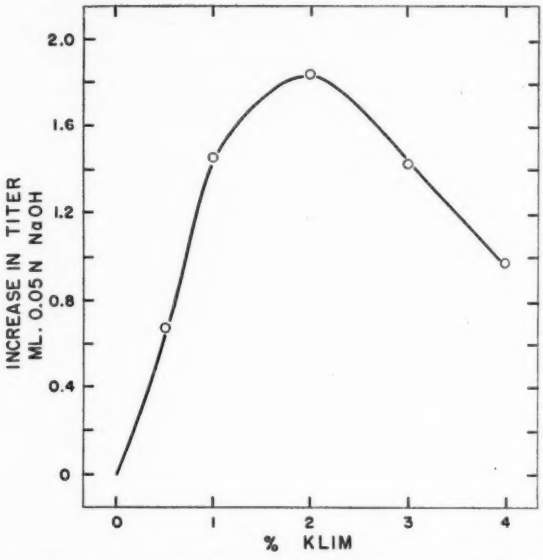

FIG. 4. *Effect of amount of Klim on production of proteases by Gliocladium PRL 79.*

to 8% resulted in the same yield of enzymes, whereas when 1% Klim was employed the maximum production of enzyme was not reached until the concentration of glucose was 8%. In media containing 2% Klim a small amount

TABLE V

EFFECT OF CONCENTRATION OF GLUCOSE AND MALT SPROUTS ON PROTEASE PRODUCTION BY *Gliocladium* PRL 79

	Increase in titer—ml. 0.05*N* NaOH			
% glucose	1% malt sprouts	2% malt sprouts	3% malt sprouts	Mean effect glucose
0	0.07	0	0	0.02
1	1.00	1.20	0.62	0.94
2	1.27	1.53	1.29	1.36
3	1.28	1.63	1.67	1.53
Mean effect of malt sprouts	0.93	1.09	0.90	

NOTE: *The media contained varying amounts of glucose and malt sprouts, mineral salts and 0.5% calcium carbonate.*

of proteases was produced without the addition of any extra carbohydrate. This may possibly be the result of the utilization of the additional lactose present in the medium.

Rate of Utilization of Nitrogen and Carbohydrate

Some attempt was made to determine whether the rate at which an organism utilized glucose had any effect on protease production. It had been noted that very rapidly growing molds such as *Aspergillus niger* PRL 326 produced their maximum amount of proteases in two days. Figs. 5 and 6 give the

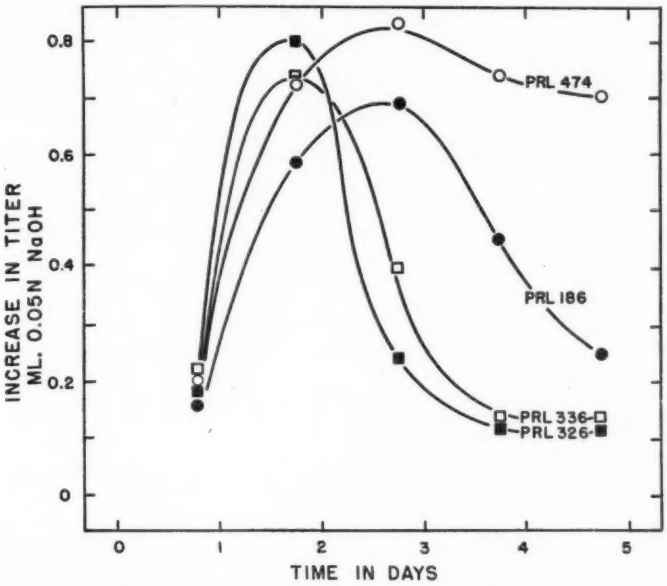

FIG. 5. *Rate of protease production by four species of Aspergillus, A. candidus PRL 474, A. fumigatus PRL 186, A. flavus-oryzae PRL 336, A. niger PRL 326.*

results of a study made on the rate of dissimilation of carbohydrate and protease production by four species of *Aspergillus*. *A. niger* PRL 326 and *A. flavus-oryzae* PRL 336 produced their maximum protease in about one and one-half days, whereas *A. candidus* PRL 474 and *A. fumigatus* PRL 186 required almost three days growth to reach their maximum. Of the two rapid protease producers *A. niger* had fermented 85% of the carbohydrate in one and one-half days, whereas *A. flavus-oryzae* which had produced the same concentration of proteolytic enzymes had only fermented 25%.

It had been assumed that the peak in enzyme production corresponded with the exhaustion of the medium, and that autolysis occurred with the consequent digestion of the proteolytic enzymes as a nitrogen source. However, the peak in enzyme concentration was often reached, as in *Gliocladium* PRL 79 and

PRL 86, when less than half the carbohydrate had been utilized. The only nitrogen in the medium was that present in Klim, and the total nitrogen available was found to have been almost completely utilized in less than two

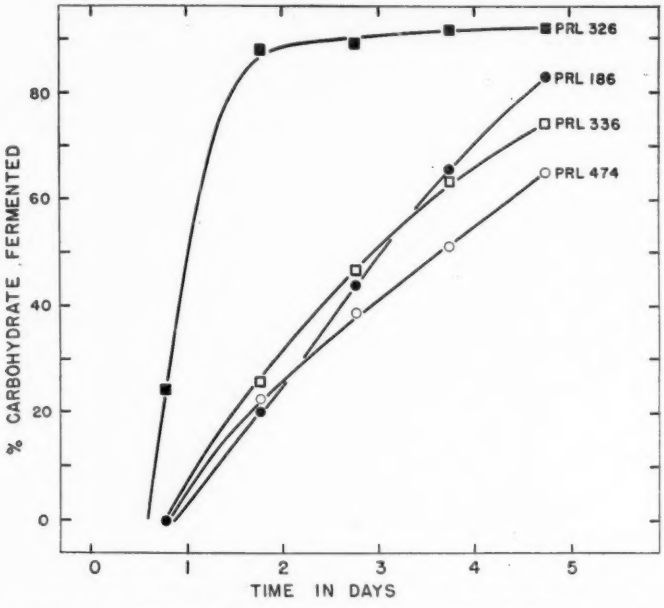

FIG. 6. *Rate of carbohydrate utilization by four species of Aspergillus. A. candidus PRL 474, A. fumigatus PRL 186, A. flavus-oryzae PRL 336, A. niger PRL 326.*

days by *Gliocladium* PRL 79, and *Streptomyces* PRL 376, and 50% utilized in the case of *Alternaria* PRL 369. Mostly in two days there was not any appreciable amount of enzyme present.

Amount of Inoculum

For the present method of submerged culture production of proteolytic enzymes it was found that a 2% inoculum of a mycelial suspension was satisfactory. Approximately 4 sq. cm. of a Petri plate culture growing on potato dextrose agar was ground with 20 ml. of sterile water, and 2 ml. of this suspension was pipetted into 100 ml. of culture medium. With cultures producing a heavy crop of conidia it was inevitable that more inoculum would be added as spores than would be added from a nonsporulating culture. Table VI shows the effect of varying the amount of inoculum from 1% to 5% of the total volume of medium, for a sporulating culture of *Gliocladium* PRL 79 and a purely mycelial culture of *Mortierella* PRL 26. It is quite evident that the amount of inoculum had no effect on protease production by these two fungi, 1 ml. containing sufficient fungal material for good growth.

TABLE VI

EFFECT OF THE AMOUNT OF INOCULUM ON PROTEASE PRODUCTION

Amount of inoculum, %	Increase in titer—ml. 0.05*N* NaOH	
	Gliocladium PRL 79	*Mortierella* PRL 26
1	1.41	0.86
2	1.51	0.80
3	1.52	0.85
4	1.46	0.78
5	1.57	0.78

Effect of the Volume of Medium in each Flask

One hundred milliliters of liquid medium was found to be the most convenient amount to handle in each shake flask. Variations in the volume of medium in each flask resulted in changes in the conditions of aeration, and Table VII shows the effect of different volumes of medium on protease production by *Gliocladium* PRL 79 and *Sordaria* PRL 257.

TABLE VII

THE EFFECT OF VOLUME OF MEDIUM ON PROTEASE PRODUCTION

Vol. per flask, ml.	Increase in titer—ml. 0.05 *N* NaOH	
	Gliocladium PRL 79	*Sordaria* PRL 257
25	1.41	0.61
50	1.12	0.94
75	1.18	0.94
100	1.36	0.92

Twenty-five milliliters of medium caused a small decrease in the yield of proteolytic enzymes from *Sordaria* PRL 257. Otherwise no significant difference in the potency of the culture filtrates was caused by variations in aeration as a result of changes in the volume of medium per flask. The concentration of enzyme per unit of medium was almost the same in all four treatments.

Reproducibility of Results

Work on the characterization of the mold enzymes was complicated by the necessity of having to work with different batches of enzymes grown at different times. The fungi showed remarkable reproducibility and stability, the enzyme concentration rarely varying by more than 10% but the actinomycetes were more variable. The proteolytic power of the *Streptomyces* isolate PRL 318 progressively deteriorated, and the culture was finally discarded

and replaced by PRL 376, which has so far shown no loss of activity after being cultured for several months, although enzyme production by this *Streptomyces* culture is not always consistent.

The yield of proteases from some of the cultures particularly PRL 79 grown during the early period of this work was not as high as later on when the conditions governing protease production in submerged culture had been more fully investigated.

Discussion

A number of unrelated fungi and actinomycetes have been shown to produce high yields of proteolytic enzymes when grown in submerged shake cultures. Although the conditions under which these enzymes are produced can be standardized to a certain extent, each organism has certain definite requirements such as time of growth, amount of nutrients in the medium, and temperature of incubation which must be satisfied before the optimum yield of enzymes from each culture can be attained.

The time required to reach the optimum concentration of proteases in the culture medium depended on the growth rate of each organism. Oshima and Church (5) stated that all enzymes disappeared from the mycelium soon after spore formation, and suggested that for this reason estimations of the proteases in the mycelium might lead to erroneous results. Berger *et al.* (1) found that the maximum amount of proteolytic enzymes was present in the mycelium at the time of spore formation. Sporulation does not occur during growth in submerged culture, and the greatest concentration of proteases in the medium was reached when the rate of mycelial production was beginning to fall off. Some attempts were made to estimate the amount of proteolytic enzymes present in the cells at this time. The mycelium certainly possessed considerable activity but comparisons between intra- and extracellular enzymes were impractical on account of the difficulty of ensuring complete extraction of the enzymes from the cells. Probably the intracellular enzymes undergo the same processes of inactivation and digestion as the enzymes in the culture medium.

It was found that neither *Gliocladium* PRL 79 nor *Alternaria* PRL 369 would produce significant amounts of proteases on media which contained a high proportion of inorganic nitrogen. Protease production by *Gliocladium* PRL 79 was almost directly proportional to the amount of protein nitrogen in the medium when this exceeded 50% of the total nitrogen. However, Crewther and Lennox (2) obtained sufficiently high yields of proteolytic enzymes from *Aspergillus oryzae* grown on a purely synthetic substrate to enable them to obtain crystals containing protease from the medium. Protease production by the aspergilli was generally so low when the molds were grown in submerged culture that little work was done on the genus.

By supplying an adequate amount of protein and carbohydrate it is possible to prolong the period during which the proteases are at their optimum concentration in the medium, and so ensure that the maximum yield of enzyme is obtained.

Waksman (7) found that when *Aspergillus niger* was grown in still culture the amount of carbohydrate present in the medium had no effect on the production of proteases. Jensen (4), however, found that fungal proteolytic activity was much greater when the molds were grown in the presence of gelatin and glucose, than when grown on gelatin alone, whereas the activity of *Actinomyces griseus* was hardly affected. He attributed this to the very poor growth made by fungi when grown on a protein substrate with no carbohydrate, and this was also found to be true of growth in submerged culture. The majority of fungi require the presence of an easily available carbohydrate source before growth and protease production are significant.

Little work was done on the effect of temperature on protease production, however the individual cultures are known to vary in their optimum temperature for growth. *Mortierella* PRL 26 produced a higher yield of proteolytic enzymes when grown at 25° C. than when grown at 30° C. The reverse was found to be true of *Gliocladium* PRL 86 which produced higher enzyme concentrations at 30° C. than at 25° C.

By a study of the requirements of individual cultures it has been found possible to increase the enzyme yield of the two *Gliocladium* cultures to almost double their original value, whereas the yields of *Mortierella* PRL 26 and *Alternaria* PRL 369 have been only slightly increased under optimum conditions.

Acknowledgment

Grateful acknowledgment is made to Dr. A. C. Blackwood, for his helpful suggestions and criticism of the manuscript.

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THE PROTEOLYTIC ENZYMES OF MICROORGANISMS

III. SOME CHARACTERISTICS OF EXTRACELLULAR PROTEASES PRODUCED IN SUBMERGED CULTURE[1]

By W. B. McConnell[2]

Abstract

Some of the general characteristics of the proteases liberated into the culture medium by molds and actinomycetes grown in submerged culture have been studied. Species of *Alternaria*, *Streptomyces*, *Mortierella*, and *Gliocladium* were used. The enzymes resemble trypsin in that they are most active at a pH slightly above 7 and are inhibited by a preparation of egg albumin. They are stable at low temperatures but suffer marked losses in activity when stored for 16 hr. above 40° C. The most rapid hydrolysis of gelatin occurs at temperatures between 40° C. and 50° C. The enzymes from different organisms show definite differences with respect to their ability to attack different proteins, gelatin and casein being in general the most readily digested. The protease systems from different organisms also vary with respect to the extent to which they can digest gelatin; some enzymes are able to release about three times as many amino groups from gelatin as others. The limit of the hydrolysis is not dependent upon substrate concentration but is slightly affected by the concentration of enzyme. The enzymes were effective in liberating free amino acids from gelatin.

Introduction

Although the proteolytic systems of animal origin have been fairly well studied, investigations about those produced by microorganisms have been limited in number and scope. The work done so far with microorganisms has indicated, however, that they are a promising source of new enzymes. It has been shown, for example, that molds produce several different proteases (3) and some success has been achieved in the separation and purification of these (4, 8).

An increase in the number and variety of proteolytic enzymes would serve many useful purposes. A suitable supply of proteases, differing widely in specificity, would be helpful to those seeking new methods of approach to the study of protein chemistry. An increase in the number of readily available and inexpensive proteases may also have a practical significance. The use of enzymatic digestion offers a number of attractive possibilities for the solubilization or modification of proteins and protein containing materials and might even prove advantageous in a few instances as an alternative to acid hydrolysis.

The present paper is a report of the findings in a preliminary study of the character of the proteolytic enzymes liberated into the culture medium by some molds and actinomycetes grown in submerged culture. After removal of the mycelium by filtration or centrifugation the medium was used directly as a source of crude protease. This method of obtaining the enzyme is a

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[2] *Chemist.*

departure from the usual procedure of studying mold enzymes as it has been customary to grow the molds in still culture and to obtain the enzyme by extraction of the mycelium. The direct use of the filtrate from the submerged culture simplifies the collection of a crude enzyme preparation, and the method can more readily be applied to larger scale production.

The work which is reported herein was done with the enzymes from five organisms: one each from the genera *Alternaria*, *Streptomyces*, and *Mortierella*, and two from the genus *Gliocladium*.

Experimental

Methods

Investigation of the character of the proteases was done entirely by observing their ability to hydrolyze different protein substrates under a variety of conditions. The formol titration, which has frequently been used as a criterion of the extent of protein hydrolysis (7, 10, 14) was employed extensively in the present work because of the rapidity with which measurements could be made. Since many of the digests were turbid or contained mold pigments it was found advantageous to use a pH meter for the purpose of determining the end point of the titration. The following procedure was adopted for all formol titrations: Five ml. of buffered protein digest was added to 45 ml. of distilled water and the mixture adjusted to pH 7 using a direct-reading pH meter employing a glass electrode. Ten milliliters of neutral 37% formaldehyde was then added and titration continued with stirring to a pH of 8.5 with standard 0.05 *N* sodium hydroxide. The increase in titer as a result of digestion was calculated in terms of nitrogen and expressed as the percentage of total substrate nitrogen. No measurable increase was observed with boiled enzymes during a period of five hours, but if digestion was to continue overnight it was necessary to prevent growth of bacteria by covering the mixtures with toluene.

It is to be noted that the results cannot be directly interpreted as per cent hydrolysis because not all protein nitrogen is bound in peptide linkage and because the rather arbitrary selection of pH values at which to begin and to end all titrations will not give a precise measure of the free amino acids for all different combinations of substrate and enzyme (14). The method did, however, give reproducible results and it was observed that a straight line relationship existed between the titer obtained and the amount of the various hydrolyzed substrates present in admixture with the fresh protein (See Table I). Since the slope of this line was not affected by the various buffers tried, the method was satisfactory for comparative work.

Table I provides a comparison between results obtained with this method and with the copper method of Pope and Stevens (13) when applied to mixtures of gelatin and acid hydrolyzed gelatin. The copper method was not extensively used because it was somewhat more cumbersome than the formol titration, involving daily preparation of the reagent, and requiring a correction for citric acid in the buffer.

TABLE I

COMPARISON OF FORMOL TITRATION WITH COPPER METHOD OF POPE AND STEVENS

Mixture of gelatin and acid hydrolyzed gelatin. Total nitrogen concentration = 1.6 mgm./ml.

% hydrolyzed gelatin in sample	Amino nitrogen liberated (% of substrate nitrogen) (In excess of blank on gelatin)	
	Formol titration	Copper method
0	0	0
25	12.5	13.1
50	25.2	26.1
75	37.6	39.4
87.5	43.8	46.3
100	50.4	54.1

Protein concentration was determined by micro-Kjeldahl estimation of total nitrogen. The free amino acids liberated during digestion were estimated by Van Slyke's manometric method (15); 2 ml. samples of the digests were boiled with 50 mgm. ninhydrin for 20 min. at a pH of 2.5 and the carbon dioxide released, measured.

Materials

The enzymes studied were produced by growing the organism in submerged culture using a Klim and glucose medium containing either calcium or magnesium carbonate. They were supplied by W. M. Dion of the fermentation section of this laboratory who has described the procedure in detail and discussed the factors affecting protease production (5).

For convenience the enzymes will be referred to by the numbers of the cultures from which they were obtained. Those used are *Alternaria tenuis* PRL 369, *Streptomyces* sp. PRL 376, the *Gliocladium roseum* spp. PRL 79 and PRL 86, and *Mortierella* sp. PRL 26.

The substrates used are listed below:

Gelatin	Difco
Casein	Fishers (soluble pure)
Edestin	Rocky Mountain Research Inc.
Peptone	Bacto, nutrient
Egg albumin	Prepared according to Kekwick and Cannan (9). Recrystallized once.
Gluten	Prepared by freeze drying of gluten dispersed in dilute acetic acid.*

* *Supplied by Dr. C. V. Lusena, Division of Applied Biology, National Research Council, Ottawa.*

Results and Discussion

The Effect of pH

The activity of the proteases at various pH's was estimated by incubating 5 ml. of the culture filtrate with 20 ml. of 1.5% gelatin and 5 ml. of buffer (0.5 *M* citric acid and 0.1 *M* disodium phosphate) at 37° C. for five hours. Fig. 1 shows the results obtained with four of the enzymes studied. Up to

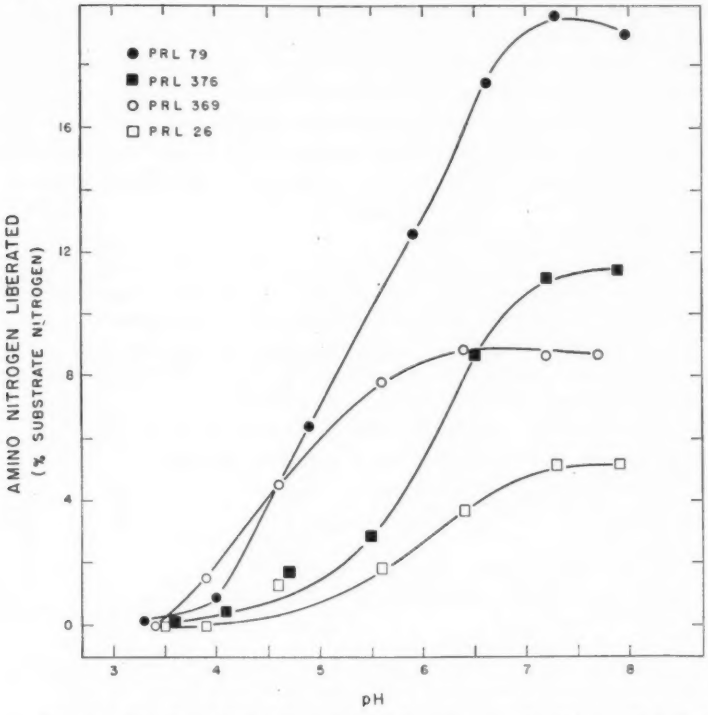

FIG. 1. *The effect of pH on the activity of the proteases. Conditions: Hydrolysis for five hours at 37° C. and pH = 7.2 with an initial gelatin concentration of 1% by weight and enzyme concentration of 16⅔% of total volume.*

pH 7 the activity increases with increasing pH, but beyond this, to the practical limit of the buffer system used, only a slight increase or a slight decrease was noted. PRL 369 was unique in that it is only slightly affected by changes of pH between 6 and 8. No activity was observed, either with the above procedure at pH 3.5, or with Anson's method for pepsin (1). Although the curves show no sharp maximum the indication of an optimum above pH 7 suggests that the enzymes resemble trypsin, an excreted protease of animal origin which is regarded as being most active at a pH near 8.

Previous reports of the pH optimum of mold proteases indicates greater differences between organisms than observed in the present work which is

concerned only with those liberated into medium during mold growth. Thibodeau and Macy (14) report the protease of the powdered mycelium of *Penicillium roqueforti* to be most active in the pH range from 5.3 to 7. Similar results were obtained by Oshima and Church (12) and Waksman (16) found that the proteases of fungi have an optimum at a reaction that is neutral or slightly acid to litmus. Berger, Johnson, and Peterson (3), in a study of 30 common molds, report that the proteinase has an optimum at about pH 7. Hecht and Civin (6) obtained a protease from yeast which they termed a trypsin, with pH optimum of 7.8. They also found a pepsin active at pH 1.8.

These enzyme systems appear to be somewhat more stable at a pH slightly lower than that at which they are most active in digestion of gelatin. In the study of factors affecting the production of protease Dion (5) found a maximum stability at a pH of about 6.5 for the same preparations of enzymes which in the present work were most active between pH 7 and 8.

The Effect of Temperature

Although the crude enzymes could be stored at temperatures near 0° C. for several weeks, it was observed that they rapidly lost activity when kept near 40° C. In order to compare the effect of heat on different enzymes they were incubated at fixed temperatures for 16 hr. in the absence of substrate and then tested for the rate at which they liberated amino groups from a 3.5% solution of gelatin buffered at pH of 7.2 and incubated at 37° C.

The results given in Fig. 2 indicate that in general an increase in storing temperature from 40° C. to 50° C. results in a drastic loss of activity and that

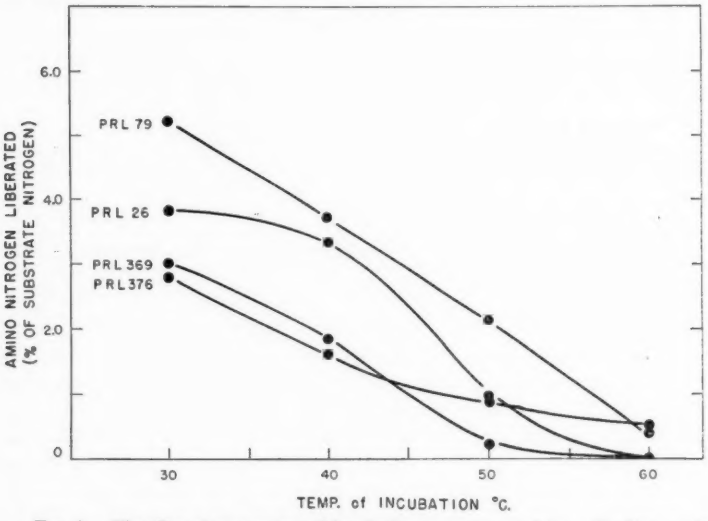

FIG. 2. *The effect of temperature of incubation on enzyme activity. Conditions: Enzymes incubated for 16 hr. at various temperatures. Tested by use for a hydrolysis at 37° C. for five hours at pH 7.2. Gelatin concentration = 3.5% by weight and enzyme concentration = 13% of total volume.*

storage at 60° C. causes almost complete destruction of proteolytic power. PRL 26 is unique in that it suffers only slight loss by a 16 hr. incubation at 40° C. PRL 369 is the most sensitive to heat as it lost all activity when held at 50° C.

The observation of a marked loss in activity as a result of incubation of the enzymes at 40° C. made it necessary to test the validity of the time–product relation when applied to these enzyme systems. This law applies when the enzyme is stable during the period of the digestion and does not contain an activator or inhibitor. It states that for a given initial substrate concentration, the product of the time required to attain a given degree of hydrolysis and the enzyme concentration is a constant. The results of experiments with PRL 26 and PRL 86 at 37° C. are given in Fig. 3 and show that the rule holds

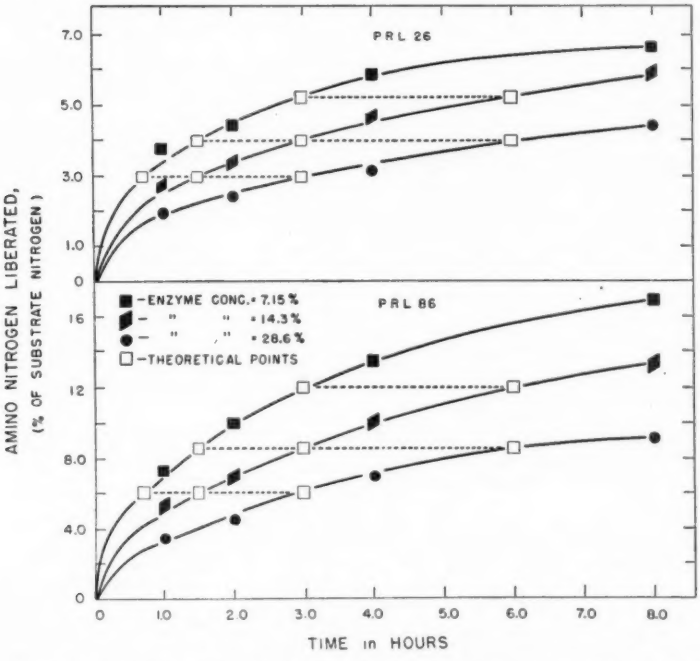

FIG. 3. *Test of the time–product relation for the enzymes. Conditions: Hydrolysis at 37° C. and pH 7.2 with initial gelatin concentration of 1.5%.*

within experimental error for the narrow limits of time and concentration used. These and other similar results indicate that there was no appreciable inactivation of the enzymes during short digestion periods. The fact that a pronounced loss of activity was observed when the enzymes were held at 40° C. in the absence of substrate suggests that the rate of thermal inactivation is less when substrate is present.

In an enzyme catalyzed reaction an increase in temperature accelerates two independent processes: the thermal inactivation of the enzyme and the reaction catalyzed by active enzyme; and as a result an optimum temperature occurs. The existence of the optimum would be as a natural consequence of having a higher temperature coefficient for the inactivation than for the reaction. Since the optimum temperature of an enzyme varies with such factors as enzyme purity, substrate, and presence of activators or inhibitors its usefulness for the purpose of characterizing the enzymes is limited. Some results obtained by digesting 3.5% gelatin solution buffered at pH 7.2 with four of the enzymes are, however, shown in Fig. 4. Amino nitrogen determinations

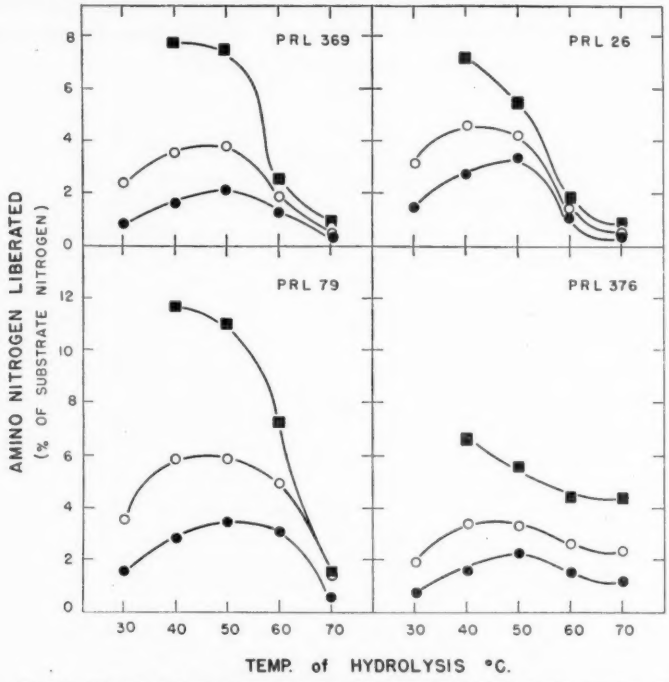

FIG. 4. *The effect of temperature on rate of hydrolysis of gelatin by the enzymes. Conditions: Hydrolysis of 3.5% gelatin using enzyme concentration of 13% total volume at a pH of 7.2. Closed circles show results of one and one-half hours hydrolysis, open circles, five hours, and squares, 24 hr.*

were made with the formol titration as described previously. The results indicate that the apparent optimum temperature for the enzymes and conditions used varies with the duration of the hydrolysis. The shift of the maximum of these curves toward lower temperatures with an increase in time is to be expected in view of the above discussion because the analyses recorded on the graphs are proportional to the total amount of hydrolysis and is therefore an integral rather than a differential function of time.

For hydrolyses of short durations a temperature near 50° C. appears to be the best, but if the hydrolysis is to be continued for five hours or longer, temperatures near 40° C. are better.

The results show that there are differences in the enzymes in the manner in which they are affected by changes in temperature. Enzymes PRL 369 and 26 show little activity after the hydrolysis has been continued for more than one and one-half hours at 60° C. while PRL 79 causes a readily measurable increase in the amount of digestion in the interval from 5 hr. to 24 hr. PRL 376 is unique in that it alone retains some activity at temperatures as high as 70° C. The stability of the protease from the *Streptomyces* isolate is in keeping with a previous report (17) that the proteolytic enzymes of actinomycetes are able to withstand temperatures of 70° C. for 30 min. They were found to remain active for long periods at 40°–45° C. but were destroyed by heating to 80° C.

The Effect of the Substrate

A large number of measurements were made involving enzymes from different organisms and with a range of substrates.

The results given in Table II are typical and show clearly that the preparations differ considerably with respect to their ability to attack different substrates. PRL 376, for example appears relatively active toward a casein

TABLE II

THE EFFECT OF SUBSTRATE ON RATE OF HYDROLYSIS

Temperature.....37° C.
Time of hydrolysis.....five hours
Enzyme concentration.....16.6% by volume
pH of substrate.....7.2
Concentration of substrate.....1%

Substrate	Increase of amino nitrogen (% of substrate N)			
	PRL 26	PRL 86	PRL 369	PRL 376
Gelatin	6.1	15.1	9.8	10.4
Casein	7.7	17.2	10.5	18.8
Gluten	3.9	10.8	4.3	0.0
Edestin	1.6	5.7	3.0	2.5
Egg albumin	0.0	0.5	0.8	1.3
Peptone	0.5	4.4	3.0	2.5

Average difference between duplicates = 0.22
Maximum difference between duplicates = 0.53

substrate but shows no ability to digest gluten while PRL 26 shows little tendency to liberate additional amino acids from the partially hydrolyzed peptone. In general, gelatin and casein are better substrates for these proteases than the others tested. The lower rate of hydrolysis of gluten may

have been partially due to the fact that the salt content of enzyme solution and buffer precipitated it, whereas all the other substrates remained in dispersion. The slow rate of attack upon edestin would not appear to be caused by the sodium chloride added to disperse it because experiments showed that similar salt concentrations did not appreciably affect the rate of digestion of gelatin.

In view of the fact that egg white is known to contain an inhibitor specific for trypsin (11) the almost complete inactivity of these proteases toward the egg albumin preparation used is interesting, because it suggests an additional similarity between these enzymes and trypsin. The inhibiting effect of the egg albumin was shown in another experiment in which its presence in the digestion mixture was found to cause a marked retardation in the rate of the digestion of gelatin by the enzyme preparations. It is of interest to note that these proteases are like trypsin in that they do not readily attack undenatured haemoglobin.

The Extent of Hydrolysis

Results obtained by prolonged digestion with the mold enzymes suggest that the extent to which they can carry the hydrolysis varies with the enzyme system. In one set of experiments using PRL 26 and 86, gelatin was digested at 37° C. for a period of about two weeks. The results (Table III) show that

TABLE III

THE EFFECT OF PROLONGED DIGESTION

Conc. enzyme, % by vol.	Conc. gelatin, % by wt.	Amino nitrogen liberated (% of substrate nitrogen)						
		3 hr.	4.3 hr.	25 hr.	48 hr.	72 hr.	123 hr.	288 hr.
PRL 86								
11.1	1	13.9	16.2	27.6	26.2	28.2	30.4	33.2
22.2	1	18.6	20.9	33.2	31.3	33.5	34.2	34.3
11.1	1.5	12.3	14.2	25.6	24.8	27.0	29.4	31.8
22.2	1.5	15.4	17.5	30.0	28.6	31.0	32.8	—
PRL 26								
11.1	1	5.3	5.9	9.2	9.0	10.0	10.6	11.5
22.2	1	6.7	7.6	10.6	9.0	9.9	10.0	9.5
11.1	1.5	4.0	5.1	8.6	8.6	9.2	10.0	11.1
22.2	1.5	5.1	6.6	9.8	8.6	9.3	9.6	10.4

there was only relatively slow hydrolysis after 48 hr. and that the final amount of hydrolysis was not markedly dependent upon either the substrate concentration or the enzyme concentration. It would appear that PRL 86 and PRL 26 can liberate about 33% and 11% of the nitrogen of gelatin as measurable by the present formol procedure. The low value obtained with PRL 26 suggests that this enzyme is quite limited in the extent to which it can hydrolyze gelatin and is probably lacking in what may be termed peptidase or

polypeptidase. This type of behavior was observed many times during the present work where it was found that during the early stages of hydrolysis PRL 26 was producing amino nitrogen at a rate comparable to or even greater than that observed for the other enzymes studied, but that the rate always decreased very markedly as digestion proceeded. When Anson's procedure for trypsin (2), which involves a five minute digestion of denatured haemoglobin, was used for estimating activity, PRL 26 appeared as potent as any of the other enzymes, an observation which led to its selection for study.

Table IV gives additional data to show the limit of the hydrolysis of gelatin by four enzymes with a variety of enzyme and substrate concentrations.

TABLE IV

THE EFFECT OF PROLONGED DIGESTION OF GELATIN WITH VARIOUS ENZYMES

Time of digestion.....three weeks
Temperature.....37° C.
pH.....7.2

Gelatin conc., % by weight	Enzyme conc., % by volume	Amino nitrogen liberated (% of substrate nitrogen)			
		PRL 79	PRL 26	PRL 369	PRL 376
2.0	37.5	41	13	37	20
1.0	37.5	43	13	39	20
0.5	37.5	44	11	35	19
2.0	12.5	38	13	35	13
1.0	12.5	35	13	38	14
0.5	12.5	37	9	31	13

The results again indicate that there is no pronounced effect due to changes in substrate concentration but that there is somewhat less hydrolysis, especially with PRL 376, when the concentration of enzyme is reduced to one-third. In view of the results obtained in the study of temperature it is to be expected that appreciable inactivation of the protease occurs during these long hydrolyses.

In connection with the lower degree of hydrolysis obtained with PRL 26 it was observed that an addition of fresh enzyme followed by incubation resulted in no measurable increase in the amount of hydrolyzed gelatin. Addition of fresh gelatin resulted in an increase in the amount of measurable amino nitrogen to a value comparable to that which would be obtained if all the substrate had been added at the initial time. Evidence that the protease from PRL 26 is not inhibited by the products of hydrolysis was obtained by addition of various amounts of a boiled hydrolyzate (gelatin digested with PRL 26) to fresh digestion mixtures of the enzyme and gelatin. The results are given in Table V.

TABLE V

EFFECT OF ADDITION OF PRODUCTS ON HYDROLYSIS RATE WITH PRL 26

Substrate.....1% gelatin
Temperature.....37° C.
Enzyme conc.....16.6% by volume
Hydrolysis time.....three hours

Hydrolysis products added (% total volume)	Increase in amino nitrogen (% of substrate nitrogen)
0	4.7
3.3	4.5
16.6	4.5
50.0	4.6

The Production of Free Amino Acids by Mold Proteases

To gain additional information about the nature of hydrolysis with these enzymes some estimates of the free amino acids in the digests were made using the ninhydrin procedure of Van Slyke.

Fig. 5 shows the results from two typical experiments in which the rate of liberation of amino groups and amino acids was measured simultaneously, and illustrates the differences between the enzymes. The sample comparison

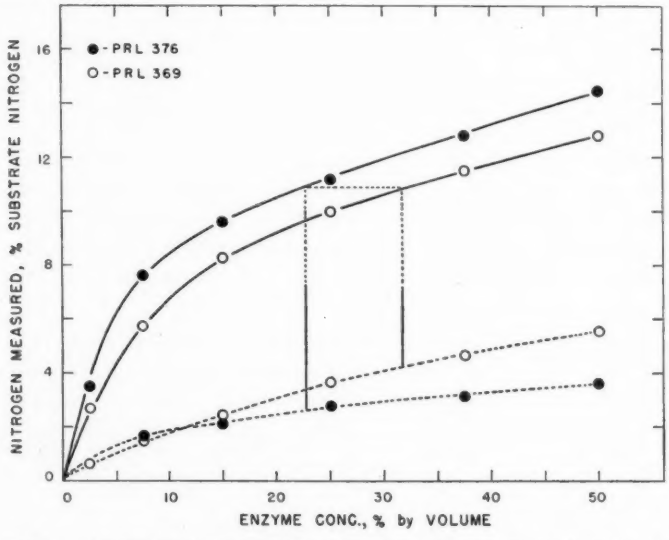

FIG. 5. *Comparison of the rate of liberation of amino groups and amino acids. Conditions: Gelatin concentration = 1%; temperature = 37° C.; hydrolysis for four and one-half hours, at pH = 7.2. Solid curves indicate free amino groups liberated, dotted curves the free amino acids liberated.*

shows that when 11% of the nitrogen has been liberated as amino nitrogen, 4.3% and 2.7% of it has appeared as free amino acids for hydrolyzates of gelatin obtained with PRL 369 and PRL 376 respectively.

In all tests an appreciable amount of nitrogen was liberated as free amino acids and as would be expected, the ratio of free amino acids to free amino groups increases with the degree of hydrolysis. The results may be summarized by saying that for digestions liberating up to 20% of substrate nitrogen as amino nitrogen, the free amino acid nitrogen constituted from 20 to 45% of amino nitrogen liberated, depending upon the extent of digestion and the enzyme used. It was observed that PRL 26 produced free amino acids in relation to free amino groups at a rate comparable to the other enzymes used. This is somewhat surprising as it was found that this enzyme was quite limited in the extent to which it would hydrolyze gelatin. More detailed study as to the specificity of the enzyme is required before an explanation is possible.

Although the results of the experiments at different pH values, together with the observation that the enzymes are inert toward an egg albumin substrate indicates that the proteases studied resemble trypsin, no other generalization can be made regarding their character. Marked differences between them are indicated by the results obtained with different substrates and at different temperatures and are also clearly shown by the variations observed in the extent to which they can hydrolyze gelatin and in the relative rates at which they liberate free amino groups and free amino acids. It is probable that these proteases, like those extracted from the mycelium, are a mixture of two or more different enzymes. More detailed investigation of the proteolytic system of the individual organisms is required to determine the number and character of the proteases present. Work based on the information obtained in this study and involving fractionation and purification procedures may supply information as to whether the differences now observed are due to different types of enzymes or to variation in the relative amounts of the same enzymes.

Acknowledgment

The author is indebted to Dr. W. M. Dion of this laboratory who supplied the proteolytic enzymes used in the work.

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PRODUCTION AND PROPERTIES OF 2,3-BUTANEDIOL

XXXVII. A STUDY OF FACTORS AFFECTING THE *BACILLUS SUBTILIS* FERMENTATION[1]

By A. C. Blackwood and F. J. Simpson

Abstract

The optimum temperature for fermentation of seven strains of the Ford type of *Bacillus subtilis* is close to 45° C. At these higher temperatures 10% glucose or equivalent hydrolyzed molasses ferments completely in six days while 10% fructose takes only three days. The rate of fermentation is similar under aerobic or anaerobic conditions but agitation or shallow layers of media increase this rate. The yields of 2,3-butanediol, acetoin, glycerol, and lactic acid are high under anaerobic conditions but in the presence of oxygen the yield of glycerol and lactic acid is decreased. The organism demonstrates strong oxidative tendencies and can give a large yield of acetoin. Other factors affecting the fermentation are also assessed.

Introduction

Some strains of the Ford type of *Bacillus subtilis* dissimilate glucose giving good yields of 2,3-butanediol and glycerol (3). The most active cultures took five days to utilize 5% glucose, but they were tested under conditions previously found (9) with one culture to give the best yields of 2,3-butanediol and glycerol. This investigation was undertaken to study some factors affecting the rate of fermentation so that optimum conditions might be established. The effects of various strains and temperatures; agitation, aerobic, anaerobic, and reduced pressure conditions; and carbohydrate concentrations and sources, were assessed in comparative tests.

Materials and Methods

Commercial cerelose was used as the main carbohydrate substrate. Where detailed analyses were made anhydrous reagent sugars were employed. The beet molasses contained 49.1% sucrose, 0.6% invert sugar, 1.46% raffinose, 0.02% amino nitrogen, 5% potassium expressed as potassium oxide, and 3.4% betaine.

The sugars were sterilized separately and sterile concentrated salts and yeast extract solutions were aseptically added after cooling. Calcium carbonate was sterilized dry in an oven at 160° C. for three hours and added in excess at the time of inoculation. The final medium contained, in addition to the sugar, 0.05% monobasic potassium phosphate, 0.05% dibasic potassium phosphate, 0.02% magnesium sulphate heptahydrate, 0.5% yeast extract, and except where otherwise indicated, 1% calcium carbonate.

Both rapid analyses by Summer's method as given by Kolmer and Boerner (6) and the more accurate method of Underkofler *et al.* (11) were used in sugar

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determinations. Sucrose determinations were made by the Lane–Eynon method (7). The molasses was cleared with potassium oxalate and activated charcoal, and hydrolyzed overnight with hydrochloric acid (10). Acetoin, 2,3-butanediol, glycerol, and ethanol were separated by ether extraction and analyzed as previously described (9). The fraction of the ether extract containing the organic acids was analyzed chromatographically (8).

Experimental Results

Effect of Temperature

Seven previously tested cultures (3) were compared. One strain produced high yields of lactic acid (No. 454) while the remaining six gave good yields of desired products. The amount of glucose utilized by these cultures after two, three, and four days' fermentation at seven specified temperatures was estimated and the results for a two-day fermentation are given in Table I.

TABLE I

EFFECT OF TEMPERATURE ON THE FERMENTATION RATE

Culture No.	Residual glucose, %						
	Incubation temperature, °C.						
	30	35	40	45	50	55	60
44	3+	3.0	2.5	0.8	1.0	3+	3+
158	3+	3+	3+	3+	3.0	3+	3+
161	3+	3+	3+	3.0	2.5	3+	3+
200	3+	3+	3.0	2.5	2.5	3+	3+
205	3+	3+	3.0	3.0	2.5	3+	3+
453	3+	3.0	2.5	1.0	3+	3+	3+
454	3+	3+	3.0	2.8	0.75	3+	3+

Conditions: Grown on 5% glucose medium for 48 hr. with 20 ml. of medium in 125-ml. Erlenmeyer flasks. Glucose analysis by Summer's method.

The optimum temperature for fermentation for all strains is at 45° C. or slightly higher. The maximum temperature for growth of this organism is not much higher and Edwards and Rettger (4) report this maximum at 54.3° C. ± 4.6°.

It appeared from some preliminary work that the relative production of some of the products at the higher temperatures of incubation differed from those formerly obtained at lower temperatures. Culture No. 453, which ferments rapidly giving high yields of desired products, was selected for detailed study of this temperature effect and as shown in Table II, the 2,3-butanediol plus acetoin to glycerol ratio appears to increase with increasing temperature. This is more evident in the 10% glucose medium than in the 5%. None of the other products appear to be similarly affected. Under anaerobic conditions

TABLE II

EFFECT OF TEMPERATURE ON THE YIELD OF PRODUCTS

	mM. product per 100 mM. glucose dissimilated									
	5% initial glucose					10% initial glucose				
	Temperature, °C.									
	30	35	40	45	47.5	30	35	40	45	47.5
Product										
Acetoin	2.52	8.58	11.90	9.96	7.58	1.81	3.46	1.26	10.95	10.44
2,3-Butanediol	56.97	55.38	50.20	52.88	58.38	59.06	62.52	65.62	58.54	57.92
Glycerol	53.45	46.51	46.22	47.05	44.11	54.94	44.93	44.93	38.70	36.43
Ethanol	11.25	11.23	9.25	10.27	8.31	8.30	10.05	10.51	10.50	9.95
n-Butyric acid	0.21	Trace	0.13	0.22	Trace	0.11	0.24	0.27	0.40	0.38
Acetic acid	0.16	4.58	2.13	1.80	1.20	0.09	0.84	1.16	3.49	3.74
Formic acid	2.00	3.52			3.09	2.73	0.29	2.00		1.34
Succinic acid	0.39	0.57	0.01	0.10	0.45	0.32	0.08	0.35	0.30	0.26
Lactic acid	6.05	8.16	7.07	9.21	8.61	8.81	6.00	5.07	3.46	5.45
Fermentation time, days	6	4	3.5	2.5	2	8	6.5	4	3.5	3
Glucose dissimilated, %	99.8	99.8	99.8	99.7	99.9	98.0	99.6	99.8	99.0	99.9
Glucose accounted for, %	95.4	98.8	94.2	97.0	97.2	97.2	97.0	98.0	97.6	96.1
(D + A)/G ratio*	1.1	1.4	1.3	1.3	1.5	1.1	1.5	1.5	1.8	1.9

* $(D + A)/G\ ratio = \frac{Diol + acetoin}{glycerol}\ ratio.$

Conditions: Culture No. 453 was grown without shaking with 150 ml. of medium in one liter Erlenmeyer flasks.

it is found that the 2,3-butanediol plus acetoin to glycerol ratio remained fairly constant at values from 0.8 to 1.0 (compare with results given in Table V).

Effect of Inoculum Amount

The total number of bacteria, as estimated by direct count with a Petroff–Hausser plate, and the yield of 2,3-butanediol and glycerol, were determined with three levels of inoculum. Samples were removed at intervals and analyzed in duplicate.

The inoculum level may be varied within wide limits (Fig. 1) with little effect on the rate of product formation or on the amount of products formed.

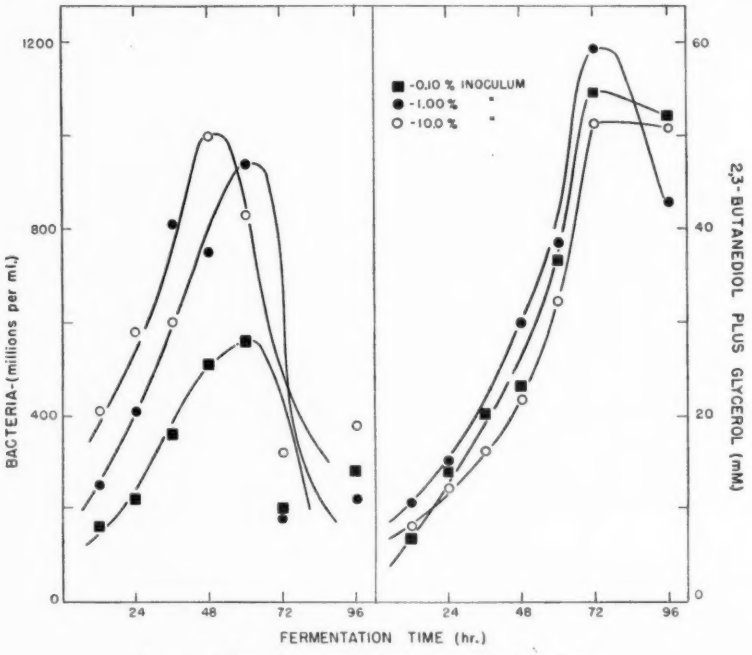

FIG. 1. *Effect of amount of inoculum on the fermentation.*

Although the number of organisms at the 10% level of inoculum was higher and reached its maximum sooner than the lower inoculum levels, the number of organisms did not appear to influence the rate of product formation. The highest yields were obtained with all inocula at 72 hr., after the number of bacteria had declined. After fermentation for 96 hr. some of the accumulated products were utilized by the organism and the total yield was lower. Similar results of variations in the inoculum level were previously reported (2) for *Bacillus polymyxa*.

Effect of Depth of Medium

In previous work it was noted that larger volumes of media with correspondingly greater depths gave slower fermentations. Katznelson (5) reported that *Aerobacillus polymyxa* fermented at greater rates and gave higher yields in media with high surface/volume ratios. Adams and Leslie (1), studying this problem, concluded that the ease of escape of carbon dioxide was a critical factor. The rate of fermentation by *B. subtilis* was also found (Table III) to be greatly accelerated in shallow layers. Four-liter bottles with perpendicular sides were used so that the surface area remained constant.

TABLE III

EFFECT OF DEPTH OF MEDIUM ON THE FERMENTATION

	Depth of medium, cm.					
	0.8	1.6	3.0	5.8	8.0	11.0
Fermentation time, days	2	3	6	7	7	7
Glucose utilized, %	95.8	98.9	99.1	75.0	59.1	62.1
2,3-Butanediol, mM.*	69.0	56.3	50.0	53.9	49.5	56.7
Glycerol, mM.*	16.4	20.0	24.0	27.6	25.8	27.5
Dissimilated glucose accounted for, %	77.2	66.3	62.0	67.6	62.4	70.0

* *mM. product produced per 100 mM. glucose dissimilated.*
Conditions: Culture No. 453 was grown at 45° C., on 10% glucose medium in four-liter bottles.

At the shallowest depth, 96% of the original glucose (10%) was fermented in two days while at the 10 cm. depth only 62% of the sugar was fermented in seven days. The percentage yields of 2,3-butanediol and glycerol varied little with the exception of the shallowest depth where relatively large amounts of 2,3-butanediol were formed. This may be explained by the greater percentage of medium in contact with air.

Effect of Agitation

The effects of aerobic and anaerobic fermentation conditions with and without shaking were determined. For agitation a B.F. Gump Co. shaker regulated at 90 revolutions per minute and imparting a gentle swirling motion was used. Anaerobic conditions were maintained by bubbling oxygen-free nitrogen through the flasks. The fermentations were done in triplicate and samples were removed at intervals from one of the flasks to determine the rate of fermentation. When glucose dissimilation was complete, detailed analyses were made on the fermented medium of the other two flasks.

Under aerobic quiescent conditions about 75% of the glucose fermented was recovered as 2,3-butanediol and glycerol, with acetoin, lactic acid, and ethanol accounting for most of the remaining sugar (Table IV). Increasing the concentration of glucose increased the 2,3-butanediol plus acetoin to

TABLE IV

EFFECT OF GLUCOSE CONCENTRATION AND SHAKING UNDER AEROBIC CONDITIONS

	mM. product per 100 mM. of glucose dissimilated							
	No agitation				Shaken			
	Initial glucose concentration, %							
	5	7.5	10	12.5	5	7.5	10	12.5
Product								
Acetoin	8.96	9.45	8.05	8.20	8.61	11.11	45.02	57.81
2,3-Butanediol	52.88	52.18	59.99	59.80	59.96	61.87	35.75	29.18
Glycerol	48.05	41.74	36.90	35.52	37.07	28.66	4.24	4.02
Ethanol	10.27	8.64	9.30	8.53	11.76	11.40	6.98	5.98
n-Butyric acid	0.22	—	0.40	0.26	0.70	1.54	2.05	1.88
Acetic acid	1.80	2.58	2.48	1.96	1.47	4.85	10.86	4.81
Formic acid	—	2.18	—	0.28	1.40	1.81	0.30	0.62
Succinic acid	0.10	0.20	0.50	0.12	0.82	0.25	0.58	0.24
Lactic acid	7.21	16.24	8.46	7.66	4.36	2.63	2.11	0.21
Fermentation time, days	2.5	5	6.5	9	1	2	5	7
Glucose dissimilated, %	99.7	99.9	98.0	99.9	99.8	99.8	99.8	99.7
Glucose accounted for, %	95.5	95.9	97.0	94.84	96.9	97.7	93.5	95.7
Ratio of acetoin + diol to glycerol	1.3	1.5	1.8	1.9	1.8	2.5	20.9	21.6

Conditions: Culture No. 453 was grown on 150 ml. of medium in one liter Erlenmeyer flasks at 45° C. with 3% calcium carbonate added.

glycerol ratio. This increase may be a result of the necessarily longer fermentation time and the influence of air diffusing into the medium rather than a direct effect of the sugar concentration. The lower levels of glucose fermented much faster, relatively, than the higher concentrations.

Shaking under aerobic conditions markedly increased the 2,3-butanediol plus acetoin to glycerol ratio. Under this oxidizing condition the amount of glycerol formed decreased with the length of the fermentation and also much of the 2,3-butanediol was converted to acetoin. As much as 87% of the glucose was converted to diol and acetoin. Somewhat larger amounts of acetic acid were produced, but little lactic acid. These effects of aerobic conditions have been previously noted (3, 9).

Anaerobic conditions favored the production of glycerol resulting in a 2,3-butanediol plus acetoin to glycerol ratio of 1.0 or lower (Table V). Under quiescent conditions this ratio declined with increasing concentrations of sugar, but on shaking this decline was not evident. Large amounts of lactic acid were produced under anaerobic conditions, especially in the shake flasks. Somewhat more ethanol was also formed.

TABLE V

EFFECT OF GLUCOSE CONCENTRATION AND SHAKING UNDER ANAEROBIC CONDITIONS

	mM. product per 100 mM. glucose dissimilated							
	No agitation				Shaken			
	Initial glucose concentration, %							
	5	7.5	10	12.5	5	7.5	10	12.5
Product								
Acetoin	4.06	0.13	0.45	1.26	1.66	1.48	1.20	1.43
2,3-Butanediol	46.17	46.58	41.85	42.73	43.03	48.18	45.67	46.30
Glycerol	48.76	56.79	60.89	59.91	45.93	46.34	50.81	48.95
Ethanol	14.78	10.32	11.77	14.93	16.12	19.69	7.16	8.71
n-Butyric acid	1.22	0.38	0.30	0.45	—	0.83	0.48	0.45
Acetic acid	1.38	1.58	2.80	0.97	0.85	1.84	1.81	1.45
Formic acid	6.05	8.02	7.33	6.60	12.08	1.64	8.67	2.77
Succinic acid	0.82	0.60	0.62	0.54	0.79	0.97	0.54	0.69
Lactic acid	22.33	31.68	28.11	25.30	42.03	24.10	37.10	37.08
Fermentation time, days	2	3	9	12	1	2	6	7
Glucose dissimilated, %	99.9	99.6	99.0	99.2	99.8	99.7	99.5	97.8
Glucose accounted for, %	95.4	97.4	94.3	95.6	97.1	98.8	95.8	96.6
Ratio of acetoin + diol to glycerol	1.0	0.8	0.7	0.7	1.0	1.0	0.9	0.9

Conditions: Culture No. 453 was grown on 150 ml. of medium in one liter Erlenmeyer flasks at 45° C. with 3% calcium carbonate added. Oxygen-free nitrogen was passed through the flask to maintain anaerobic conditions.

Agitation under both aerobic and anaerobic conditions markedly increased the rate of fermentation.

Fermentation of Various Substrates

Glucose, fructose, and sucrose were fermented under aerobic conditions at 45° C. Fructose was found to be dissimilated at a faster rate than glucose, and glucose faster than sucrose (Table VI). There was a relatively large amount of acetic acid formed when sucrose was the substrate and much of the diol was converted to acetoin. The slow rate of fermentation and the aerobic conditions probably account for these differences.

The production of 2,3-butanediol and glycerol on a commercial scale would require a cheap source of fermentable carbohydrate, such as beet molasses. A number of trial fermentations were made to determine whether beet molasses might prove a suitable substrate. The molasses was accurately weighed into one liter Erlenmeyer flasks and the sucrose content determined from a control.

TABLE VI

FERMENTATION OF DIFFERENT SUGARS

	mM. product per 100 mM. of C_6 sugar dissimilated					
	Glucose		Fructose		Sucrose	
	Initial sugar concentration, %					
	5	10	5	10	5	10
Product						
Acetoin	6.20	9.45	9.92	1.12	16.95	26.83
2,3-Butanediol	55.43	57.95	52.54	65.40	36.27	32.67
Glycerol	49.65	37.75	53.82	49.90	47.74	42.27
Ethanol	8.92	8.64	9.43	9.28	10.29	9.33
n-Butyric acid	0.38	0.37	0.38	0.51	2.13	2.98
Acetic acid	2.01	2.58	2.94	0.78	16.67	16.98
Formic acid	—	0.83	—	1.74	1.17	2.09
Succinic acid	0.25	0.35	Trace	0.48	0.36	0.46
Lactic acid	10.35	7.96	4.48	5.47	8.52	8.72
Fermentation time, days	2.5	6	1.5	3	4	9
Sugar dissimilated, %	98.0	98.6	99.0	97.5	95.2	96.6
Sugar accounted for, %	97.3	96.0	97.7	99.9	94.3	98.6
Ratio of acetoin + diol to glycerol	1.2	1.8	1.2	1.4	1.1	1.4

Conditions: Culture No. 453 was grown aerobically at 45° C. with 150 ml. of medium in one liter flasks without agitation.

The molasses was then treated by a number of different methods and the final volumes adjusted so that each flask contained 150 ml. of a 20% molasses medium (10.10% sucrose).

The fermentations were divided into three sets, the first acting as a control with an unchanged pH of 9.4, the second was neutralized with sulphuric acid to a pH of 5.5 which, during autoclaving, rose to a pH of 6.4, and the third set was hydrolyzed by reducing the pH to 3.0, autoclaving for 10 min., and readjusting the pH with sterile sodium hydroxide to approximately 6.5. Three flasks of each set were inoculated and fermented without nutrients, while yeast extract, potassium phosphate, and magnesium sulphate, as previously described, were added to a second set. All flasks contained 1% calcium carbonate.

The results are given in Table VII. The addition of nutrients appears essential for a complete fermentation. Only where the molasses was acid hydrolyzed and where nutrients were added was the fermentation rate satisfactory. Under these conditions 10% sucrose was fermented completely in six days giving a yield of acetoin, 2,3-butanediol, and glycerol which accounted for 49% of the fermented substrate. The comparatively lower yield

TABLE VII

FERMENTATION OF MOLASSES

	mM. product per 100 mM. C_6 sugar dissimilated			
	Untreated	Neutralized	Molasses prehydrolyzed	
Product				
Acetoin	11.30*	9.84*	28.13	23.45*
2,3-Butanediol	35.20	40.00	29.00	32.01
Glycerol	46.00	45.32	37.58	37.92
Ethanol	4.36	4.06	4.13	6.96
n-Butyric acid	1.24	1.00	0.93	1.02
Acetic acid	26.54	15.36	11.47	24.09
Formic acid	0.70	1.06	0.86	0.14
Succinic acid	0.45	0.35	0.75	0.42
Lactic acid	5.64	4.08	12.78	3.62
Fermentation time, days	18	16	20	6
Sucrose dissimilated, %	95.5	93.4	97.0	98.5
Sucrose accounted for, %	84.9	82.9	89.6	89.0
Ratio of acetoin + diol to glycerol	1.0	1.1	1.5	1.5

* *Yeast extract and salts added to the molasses medium.*

Conditions: Culture No. 453 was grown in 150 ml. of medium (20% molasses, 10.1% sucrose) in one liter flasks at 45° C. without agitation. Where no nutrients were added to the untreated medium and the neutralized medium, the fermentation did not go to completion in 27 days.

of glycerol could be prevented by fermenting anaerobically. The rate of fermentation is comparable to that of glucose under similar conditions. Beet molasses, when hydrolyzed and supplemented with added nutrients, appears to be a favorable substrate for this fermentation.

Discussion

The dissimilation of sugars by these Ford type strains of *Bacillus subtilis* is complex and very sensitive to fermentation conditions. The best yields of diol and glycerol previously reported (3) have not been increased but the rate of fermentation has been greatly accelerated. The use of the high optimum temperature will be advantageous in larger scale fermentations as some contamination difficulties will be obviated. The optimum conditions for a high yield of the desired products are shown to include a temperature of 45° C., 5–10% fermentable carbohydrate, nutrients, anaerobic conditions, and agitation. The fermentation obtained with treated molasses indicates that this relatively cheaper carbohydrate source is satisfactory. Further studies will probably indicate a better treatment for this molasses medium.

This organism is more strongly oxidative than any of the 2,3-butanediol producers previously studied. Very high yields of acetoin have been obtained (58% of theory) and the yield undoubtedly could be increased by aeration of

the medium, if the product were desirable. Apparently the 2,3-butanediol is easily oxidized to acetoin but the latter product is oxidized only slowly or not at all. The large amounts of acetoin that accumulate do not appear to be deleterious to the rate of fermentation by this culture.

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ANTIBIOTIC XG AS A SEED TREATMENT FOR THE CONTROL OF LEAF AND POD SPOT OF PEAS CAUSED BY *ASCOCHYTA PISI*[1]

By V. R. Wallen[2] and A. J. Skolko[3]

Abstract

Antibiotic XG has been studied for its possible application as a pea seed treatment in the control of *Ascochyta* leaf and pod spot. Fungistatic properties of Antibiotic XG against *Ascochyta Pisi* at various concentrations in relation to pH and temperature *in vitro* were studied. At antibiotic concentrations up to 1 p.p.m. no fungistatic effect was observed at pH values from 3.5 to 6.5, but, at concentrations above 1 p.p.m., complete inhibition of growth was found at all pH values tested. In the temperature range of 22° C. to 30° C. antibiotic concentrations of 1 p.p.m. had no effect on growth. Fungicidal activity to spores of *A. Pisi* has also been tested. A spore suspension of 10,000 spores per ml. required an exposure of 12 hr. with an antibiotic concentration of 100 p.p.m. for complete lethal dosage. The soaking of diseased pea seed samples in Antibiotic XG at a concentration of 25 p.p.m. for a period of 18 hr. effectively controls *A. Pisi* infection as determined by laboratory test and does not seriously impair germination. This treatment is compatible with Spergon applied after the seed has been air-dried. Storage of treated seed for a period of three months did not affect the degree of control but reduced the germinative capacity in laboratory plate tests.

Introduction

Undoubtedly the most serious seed-borne disease of peas is that commonly referred to as *Ascochyta* blight. Losses result from reduced germination and stand, lower yields, and poor quality of seed. In extreme cases total crop failure may occur. Three related fungus species are involved, namely, *Mycosphaerella pinodes* (Berk. and Blox.) Vestergr. causing mycosphaerella blight, *Ascochyta pinodella* Jones causing ascochyta footrot, and *Ascochyta Pisi* Lib., causing a disease for which the common name of ascochyta leaf and pod spot has been proposed (15). Of the three diseases, the one caused by *Mycosphaerella pinodes* is reported as being the most damaging in the United States, (11, 15, 24). In Sweden (9), *A. Pisi* is the most prevalent on seed but the amount of damage caused in the field is not known. Analyses of pea seed samples in the U.S.S.R. indicated that *A. Pisi* and *M. pinodes* were responsible for the two major pea diseases (5). Under field conditions in eastern Canada, *A. Pisi* appears to be at least as important as *M. pinodes*. Although all three species are seed-borne, an examination of several hundred samples of Canadian-grown pea seed over a period of 10 years has shown that *A. Pisi* is by far the most common seed-borne one of the three.

In the control of ascochyta blight, main reliance has been placed on the use of disease-free seed, usually from areas in western United States where semi-arid conditions unfavorable for disease development exist, and on certain

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cultural practices, such as sanitation, crop rotation, and isolation. No commercially available resistant varieties are known, but it is claimed (9) that early maturing varieties suffer less than late maturing varieties. Chemical seed treatment has not proved successful because of the deep-seated nature of infection (9, 12, 15, 21, 26), although some benefits from seed treatment have been claimed. Leach and Snyder (16) found that the application of seed-treating fungicides did effectively control surface-borne inoculum of *A. pinodella*. On the basis of numerous seed samples examined, we have come to the conclusion that surface-borne inoculum constitutes only a minor source of infection. Unless the crop is harvested under wet conditions, there would likely be little surface contamination of the seed by spores that are dependent on moisture for their dispersal. In view of the results obtained by Leach and Snyder (16), chemical seed treatment can be expected to eliminate this source of infection. The fact that seed infection normally takes place through the pod would suggest that mycelial invasion of the seed, with resulting internal infection, is the more common means of infection. The successful use of a hot-water treatment for controlling deep-seated infection has recently been reported by Gadd (9), but hard seeds, which remain unswollen in the presoak period, did not respond to this treatment and, therefore, constitute a potential source of infection.

As none of the chemical seed treatments appear to be effective in controlling ascochyta blight, it was decided to investigate the possibility of using certain antibiotic substances. Previous reports of seed treatment with antagonistic organisms, their metabolic products, or with known antibiotic substances have been concerned with their protective value against soil-borne pathogens or with their disinfestive properties against surface-born pathogens. Simmonds (22) and Ledingham *et al.* (17) found that the bacterial flora occurring on wheat kernels was antagonistic to *Helminthosporium sativum*. The bacterization with mycolitic species of *Achromobacter* and *Pseudomonas* for the protection of wheat and flax seedlings from species of *Fusarium*, *Colletotrichum*, and other fungi was used by a number of Russian investigators (3, 4, 6, 7, 19, 20). Goodman and Henry (10) reported that subtilin reduced infection when applied to barley seed that had been artificially infected with *Xanthomonas translucens cerealis* before treatment. Ark (1, 2) showed that streptomycin could be used to disinfect cucumber seed that had been artificially contaminated with *Pseudomonas lachrymans*. Darpoux (8) disinfected tobacco seed, which had been inoculated with *Pseudomonas tabaci*, by steeping the inoculated seed in the unfiltered cultural medium of an Actinomycete. No definite instance, however, is known in which antibiotic substances, used as seed treatments, have been effective in disinfecting seed of an internally established pathogen. Timonin (23) found that patulin was ineffective in controlling loose smut of wheat, although at dilutions of 1 : 20,000 it completely inhibited *in vitro* the growth of *Ustilago Tritici*. Wallen *et al.* (25) found that actidione at concentrations as high as 225 p.p.m. failed to control *Ascochyta Pisi* in infected peas.

A number of antibiotic substances, reported as possessing antifungal properties, were tested against *Ascochyta Pisi* in order to select the most promising of them. Of the several antibiotics tested Antibiotic XG was selected for further study as a possible seed treatment for the control of ascochyta leaf and pod spot. Antibiotic XG was first isolated from the filtrate of an organism closely related to *Bacillus subtilis* by Lewis, Hopper, and Shultz (18). Johnson and Burden (14) had previously isolated a similar antibiotic substance from *Bacillus subtilis* called eumycin, and found it to be active against *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, and some of the higher fungi. Hobby *et al.* (13) have shown that Antibiotic XG possesses both fungistatic and fungicidal properties to certain pathogenic fungi.

Effect *in vitro* of Antibiotic XG on the Growth of *A. Pisi*

Fungistatic Action

Preliminary tests by means of the agar streak method of antibiotic assay showed that the growth of *A. Pisi* was inhibited on pea agar containing XG above 1 p.p.m. at a temperature of 24° C. and a pH of 5.0. The effect of the pH on the fungistatic activity of XG was then ascertained by using pea agar plates at pH 3.5, 4.5, 5.2, 5.5, and 6.5 with XG concentrations of from 0 to 100 p.p.m., streaking the plates with a spore suspension of *A. Pisi*, and incubating at 24° C. It may be seen in Table I that the pH of the medium has

TABLE I

FUNGISTATIC ACTIVITY OF ANTIBIOTIC XG ON *A. Pisi* IN RELATION TO pH

Antibiotic XG concentration, p.p.m.	Growth of *A. Pisi* on pea agar at 24° C. at pH:				
	3.5	4.5	5.2	5.5	6.5
0	+	+	+	+	+
0.1	+	+		+	+
0.5	+	+		+	+
1.0	+	+	+	+	+
2.0	−	−		−	−
3.0	−	−		−	−
4.0	−	−		−	−
5.0	−	−	−	−	−
15.0	−	−	−	−	−
25.0	−	−	−	−	−
50.0	−	−	−	−	−
100.0	−	−	−	−	−

\+ *Denotes that growth occurred.*
− *Denotes that no growth occurred.*

little effect on the fungistatic properties of XG within the values tested. At concentrations up to 1 p.p.m., no fungistatic effect was noted, but, at concentrations above 1 p.p.m. complete inhibition of growth was found at all pH values tested.

The possible effect of temperature on the fungistatic activity of XG was determined in a similar way. The results are tabulated in Table II. Within the normal range for growth of *A. Pisi*, temperature has no apparent effect on XG.

TABLE II

EFFECT OF TEMPERATURE ON FUNGISTATIC ACTIVITY OF ANTIBIOTIC XG AS INDICATED BY GROWTH OF *A. Pisi*

Antibiotic XG concentration, p.p.m.	Temperature			
	22° C.	26° C.	30° C.	37.5° C.
0	+	+	+	−
1	+	+	+	−
5	−	−	−	−
15	−	−	−	−
25	−	−	−	−
50	−	−	−	−
100	−	−	−	−

+ *Denotes that growth occurred.*
− *Denotes that no growth occurred.*

TABLE III

FUNGICIDAL ACTION OF ANTIBIOTIC XG ON SPORES OF *Ascochyta Pisi*

Spore density, No. per ml.	Time of exposure, hr.	Antibiotic XG concentration, p.p.m.	Colony count in Petri plates
10,000	6	0	176
"	"	5	80
"	"	15	100
"	"	25	105
"	"	50	50
"	"	100	55
10,000	12	0	∞*
"	"	5	81
"	"	15	33
"	"	25	—
"	"	50	6
"	"	100	0
60,000	6	0	181
"	"	5	128
"	"	15	130
"	"	25	114
"	"	50	105
"	"	100	75

* *Colonies numerous and fused; count not possible.*

Fungicidal Action

In preliminary test for fungicidal activity of Antibiotic XG on spores of *A. Pisi*, it was found that, with a spore suspension of density of about 10

million per ml., spore germination was retarded, but the fungicidal level was not attained with a concentration of 100 p.p.m. and an exposure of 24 hr. Hobby *et al.* (13) found that, with more than 10,000 organisms per ml., no fungistatic or fungicidal action by Antibiotic XG on *Monilia albicans* was evident at 26° C.

Spore suspensions of 10,000 and 60,000 per ml. were prepared and placed in XG concentrations of 0, 5, 15, 25, 50, and 100 p.p.m. for six hours. A suspension of 10,000 per ml. was also exposed for 12 hr. After the spores were removed from the antibiotic solution by centrifuging, they were washed twice in sterile water, plated on pea agar and incubated at 24° C. (Table III).

It may be seen in Table III that complete fungicidal effect was attained only with a concentration of XG between 50 and 100 p.p.m. in a spore suspension of 10,000 per ml. after exposure for 12 hr.

Toxicity of Antibiotic XG to Pea Seed Germination

To determine the maximum concentration and soaking period that could be used in treating pea seed without phytotoxic effect, the following tests were made.

Filter Paper Test

Concentrations of 0, 1, 5, 15, 25, 50, and 100 p.p.m. of Antibiotic XG were prepared and 5 ml. of each concentration was flooded on filter paper in Petri dishes. Seed of the variety Valley, which had been found by laboratory plate test to be free of *Ascochyta* and other pathogenic fungi, was surface sterilized with chlorine and placed on the filter paper. Five seeds were placed in each dish and duplicated for each concentration. The dishes were then incubated at room temperature for a period of 10 days.

In the plates to which no XG had been added, germination and growth of the pea seedlings were normal. In all the plates to which XG had been added, even at a concentration of 1 p.p.m., severe toxic effects were to be noted in the germinating pea seeds. Almost complete inhibition of growth of the epicotyl and failure of lateral roots to develop were observed. It must be noted, however, that in a test such as this, the seed is subjected to a continuous exposure with increasing antibiotic concentration as a result of evaporation, and, therefore, the test is hardly comparable to a discontinuous exposure at a constant concentration in a seed treatment. Seed treatment involving the soaking of seed in an antibiotic solution, drying, and storage might be expected to result in increased antibiotic concentration and injury. Some evidence of this was found in tests reported later.

Agar and Soil Tests

Clean seed of the variety Valley was soaked in Antibiotic XG concentrations of from 0 to 100 p.p.m. in the proportion of 120 ml. of solution to 100 seeds for a period of 6, 12, 18, or 24 hr. and was then allowed to dry at room temperature

for a period of from four to seven days before sowing. For the agar test, 100 seeds were used for each concentration and each exposure period. They were treated with 2% chlorine solution to remove surface contamination and plated on pea agar, five seeds to a plate. The plates were incubated for 10 days at 24° C. For the soil test, 200 seeds were used for each concentration and exposure. They were planted in flats of sterilized soil in the greenhouse, 100 seeds to a flat (Table IV).

TABLE IV

PERCENTAGE OF GERMINATION ON AGAR AND STERILIZED SOIL OF VALLEY PEA SEED TREATED WITH ANTIBIOTIC XG

Period of soaking, hr.	Antibiotic XG concentration, p.p.m.	Germination, %	
		On agar	In soil
6	0	72	79
6	25	60	86
6	50	45	79
6	75	40	71
6	100	22	35
12	0	52	83
12	25	41	74
12	50	12	53
12	75	8	26
12	100	2	12
18	0	81	78
18	25	80	75
18	50	72	46
18	75	13	15
18	100	11	10
24	0	82	83
24	25	75	79
24	50	44	40
24	75	11	9
24	100	6	4

Table IV shows that no appreciable reduction in germination in soil occurred in the six hour treatment below 100 p.p.m. concentration, whereas 12, 18, and 24 hr. treatments showed markedly lower germination at 50 p.p.m. and higher. On the basis of soil germination, a concentration of 25 p.p.m. for 12 to 24 hr. could be used. For some unknown reason, germination on agar after 6 and 12 hr. treatment was low even in the checks, but after 18 and 24 hr. treatment the results were essentially the same as in soil.

Fungicidal Efficiency of Antibiotic XG Against *Ascochyta Pisi* in Pea Seed

To determine the effectiveness of Antibiotic XG in controlling ascochyta leaf and pod spot in internally infected seed, the agar plate test, described above, was considered more reliable than a soil test. In agar plates, when seed fails to germinate or the seedlings show no disease symptoms after seed

treatment, the fungus will develop abundantly on the agar and be easily identifiable if it is still present in a viable state. In the soil test, on the other hand, when the seed is heavily infected, the seedling may not emerge. Infected seedlings that do emerge from the soil may show no early symptoms of disease and symptoms that appear later may be the result of infection other than from the seed. However, soil tests were made for the purpose of determining whether XG treatment of seed seriously affected germination under field conditions. The seed used was taken from two samples of Lincoln peas that had been found by plating test to be internally infected with *Ascochyta Pisi*. One sample of seed was grown at Ottawa, Ont., and had been found to be infected by *A. Pisi* to the extent of 14%. The second sample was grown at Grande Prairie, Alta., and had 15.5% infection in the seed. Both seed samples were used in the agar plate tests but only the Grande Prairie seed was used in the field tests.

Agar Plate Tests

The Ottawa seed was treated with Antibiotic XG for 6, 12, 18, and 24 hr. at concentrations of 0, 25, 50, 75, 100, and 200 p.p.m. The Grande Prairie seed was treated for 18 and 24 hr. with the same antibiotic concentrations. Immediately after treatment, the seed was dried at room temperature, treated with 2% chlorine solution to remove surface contamination, and plated on pea agar, five seeds to a plate. The plates were incubated for 10 days at 24° C. The results are tabulated in Table V.

Table V shows that, for the Ottawa seed, treatments of 6 and of 12 hr. failed to control *A. Pisi*, except at antibiotic concentrations that seriously impaired germination. A treatment of 18 hr. with a concentration of 25 p.p.m. gave excellent control, with little reduction in germination, but, at 50 p.p.m., germination was depressed. In the 24-hr. treatment series, excellent control of *A. Pisi* was obtained but germination was again reduced. In the series with the Grande Prairie seed, the percentage of infection in the checks was much higher than previous tests indicated (15.5%). The reason for this marked difference is not known, but here again an 18 hr. treatment at 25 p.p.m. greatly reduced infection without affecting germination. Those seeds from which *A. Pisi* was recovered after treatment failed to germinate. Under field conditions, seedlings from these seeds would probably fail to emerge and, therefore, would not constitute a source of secondary infection in the crop.

A further test of the ability of Antibiotic XG to control *Ascochyta Pisi* infection in pea seed was made by selecting from the Grande Prairie sample, seeds that were discolored and lesioned. Some of these seeds were so badly infected that they were deformed. By plating test, the selected seed was found to be infected with *A. Pisi* to the extent of 39%. One hundred seeds from the selected sample were treated with Antibiotic XG in a concentration of 25 p.p.m. for 18 hr., dried, treated with chlorine, and plated on pea agar as before. *A. Pisi* was recovered from only 7% of the seeds, and all of these failed to germinate. Germination in the sample as a whole was only 20%.

TABLE V

FUNGICIDAL EFFICIENCY OF ANTIBIOTIC XG AGAINST *A. Pisi* IN LINCOLN PEAS, AS DETERMINED BY THE AGAR PLATE TEST

Source of seed	Number of seeds	Period of treatment, hr.	Antibiotic XG concentration, p.p.m.	Germination, %	*Ascochyta Pisi*, %
Ottawa	200	6	0	79	14
"	100	6	25	77	14
"	200	6	50	76	11
"	100	6	75	51	7
"	200	6	100	43.5	8
"	200	6	200	11	3
Ottawa	100	12	0	74	13
"	100	12	25	73	12
"	100	12	50	66	9
"	100	12	75	36	5
"	100	12	100	23	4
"	100	12	200	0	0
Ottawa	100	18	0	68	11
"	100	18	25	67	0
"	100	18	50	48	1
"	100	18	75	22	0
"	100	18	100	4	1
"	100	18	200	0	0
Ottawa	200	24	0	58.5	6
"	100	24	25	43	0
"	200	24	50	48	1
"	100	24	75	3	1
"	200	24	100	13.5	0.5
"	200	24	200	0	0
Grande Prairie	400	18	0	63.8	37.3
"	400	18	25	67.5	4.5
"	400	18	50	49	2.0
"	200	18	75	43	1.5
Grande Prairie	200	24	0	63.5	51.5
"	200	24	25	56.5	2.0
"	200	24	50	54.0	3.5
"	200	24	75	33.5	0.5

In order to determine whether Antibiotic XG in the treatment of pea seed produced only a temporary fungistatic check of *A. Pisi* or whether its action provided a more lasting control, seed of the Grande Prairie sample was treated as before at concentrations of 0, 25, and 50 p.p.m. for 18 hr. and, after drying, was stored at room temperatures for a period of three months. Two hundred seeds treated at each concentration were then plated on pea agar with the results as shown in Table VI.

Soil Tests

It was shown earlier (Table IV) that seed treatment of clean seed of Valley peas with Antibiotic XG in sterilized soil in the greenhouse did not affect emergence when antibiotic concentrations of 25 p.p.m. were used even for as long as

TABLE VI

EFFECTIVENESS OF ANTIBIOTIC XG AGAINST *A. Pisi* IN LINCOLN PEA SEED THREE MONTHS AFTER TREATMENT

Antibiotic XG concentration, p.p.m.	Period of treatment, hr.	Germination, %	*Ascochyta Pisi*, %
0	18	66	13
25	18	53	0
50	18	32	0.5*

* *Seed failed to germinate.*

24 hr., but, at concentrations of 50 p.p.m. or higher, emergence was reduced except at short exposures of six hours. Similar tests were conducted with diseased seed of Lincoln peas from the Grande Prairie sample, emergence counts being made after 18 days. The results are presented in Table VII.

TABLE VII

PERCENTAGE OF EMERGENCE AND OF INFECTED SEEDLINGS FROM INFECTED LINCOLN PEA SEED TREATED WITH ANTIBIOTIC XG AND PLANTED IN STERILIZED SOIL

Antibiotic XG concentration, p.p.m.	Period of treatment, hr.	Emergence, %	Infected seedlings, %
0	6	86	10
25	6	80	3
50	6	74	3
75	6	75	2
100	6	60	2
0	12	78	5
25	12	77	1
50	12	73	1
75	12	66	2
100	12	40	0
0	18	68	3
25	18	75	1
50	18	80	0
75	18	69	0
100	18	59	0
0	24	85	4
25	24	71	0
50	24	76	0
75	24	56	0
100	24	41	0

In Table VII it is evident that treatment with Antibiotic XG did not affect germination of the diseased seed sample grown in sterilized soil in the greenhouse when the antibiotic concentration was 50 p.p.m. or less and, with an 18 hr. exposure, disease control was good.

In order to determine the effect of Antibiotic XG treatment under field conditions and at the same time to establish the compatibility of Antibiotic XG with Spergon applied as a seed protectant, extensive field tests were conducted, the following treatments being used:

A. Seed unsoaked; without Spergon.

B. Seed unsoaked; treated with Spergon.

C. Seed soaked in water for 18 hr.; without Spergon.

D. Seed soaked in water for 18 hr.; treated with Spergon.

E. Seed soaked in XG concentration of 25 p.p.m. for 18 hr.; without Spergon.

F. Seed soaked in XG concentration of 25 p.p.m. for 18 hr.; treated with Spergon.

G. Seed soaked in XG concentration of 50 p.p.m. for 18 hr.; without Spergon.

H. Seed soaked in XG concentration of 50 p.p.m. for 18 hr.; treated with Spergon.

The seed was sown at the rate of 100 seeds per rod row, and the rows were randomized, each treatment being replicated 14 times.

In the first series of field tests, diseased Lincoln seed from Grande Prairie, the same sample as that of the previous tests, was used. The seed was sown on May 12, and emergence counts made on June 2. Following planting, the weather remained cold and dry. There was no precipitation for a period of two weeks and conditions for germination were extremely unfavorable during this period. The average percentage of emergence following the various treatments is presented in Table VIII.

TABLE VIII

PERCENTAGE EMERGENCE IN LINCOLN PEA SEED TREATED WITH ANTIBIOTIC XG AND AFTERWARDS WITH SPERGON IN FIELD SOIL UNDER CONDITIONS UNFAVORABLE FOR EARLY GERMINATION

Seed treatment	Emergence, %
A. Unsoaked; without Spergon	57.3
B. Unsoaked; Spergon treated	70.4
C. Soaked in water; without Spergon	55.2
D. Soaked in water; Spergon treated	63.4
E. XG, 25 p.p.m., 18 hr.; without Spergon	45.7
F. XG, 25 p.p.m., 18 hr.; Spergon treated	52.8
G. XG, 50 p.p.m., 18 hr.; without Spergon	7.5
H. XG, 50 p.p.m., 18 hr.; Spergon treated	14.2

Least significant difference, 4.9.

In view of the unfavorable conditions for germination in the first test, the experiment was repeated on June 9, being replicated six times. Following planting, weather conditions were much more favorable for germination than in the first test. Emergence counts were made on June 28. The results are presented in Table IX.

TABLE IX

PERCENTAGE EMERGENCE IN LINCOLN PEA SEED TREATED WITH ANTIBIOTIC XG AND AFTERWARDS WITH SPERGON IN FIELD SOIL, UNDER CONDITIONS FAVORABLE FOR GERMINATION

Seed treatment	Emergence, %
A. Unsoaked; without Spergon	56.8
B. Unsoaked; Spergon treated	67.7
C. Soaked in water; without Spergon	51.2
D. Soaked in water; Spergon treated	62.8
E. XG, 25 p.p.m., 18 hr.; without Spergon	51.3
F. XG, 25 p.p.m., 18 hr.; Spergon treated	62.7
G. XG, 50 p.p.m., 18 hr.; without Spergon	42.2
H. XG, 50 p.p.m., 18 hr.; Spergon treated	55.7

Least significant difference, 8.1.

In general, the results presented in Tables VIII and IX are quite comparable. In both experiments, unsoaked seed treated with Spergon gave significantly higher emergence than unsoaked seed that was not treated with Spergon. Soaking the seed in water did not seriously reduce emergence. Spergon treatment of soaked seed had a beneficial effect on the emergence.

As shown in Table VIII, treatments with Antibiotic XG at 25 and 50 p.p.m. resulted in significantly lower emergence than in the seed that was not treated with XG. Although treatment with Spergon increased the emergence, it did not increase it sufficiently to compensate for the low emergence. It was considered possible, because of unfavorable weather conditions for germination, that the long period that had elapsed between the time the seed was planted and the time it germinated may have resulted in increased Antibiotic XG concentration within the seed and consequent injury to the embryo. The results presented in Table IX support this contention. Emergence following an Antibiotic XG treatment of 25 p.p.m. for 18 hr. was almost identical with that of the soaked checks and not significantly lower than the unsoaked check. The Spergon treatment appears to be compatible also with the Antibiotic XG treatment. At a concentration of 50 p.p.m. for 18 hr. the treatment with Antibiotic XG resulted in low emergence.

Since, in the above experiments, a garden variety of peas was used, it seemed desirable to determine the effect of an Antibiotic XG seed treatment upon field emergence of a field variety of peas. An uninfected sample of Valley peas was selected because a sufficiently large sample of infected field

peas was not available. The seed was treated as in the previous test and planted when conditions for germination were favorable. Each treatment was replicated four times. The results are tabulated in Table X.

TABLE X

PERCENTAGE EMERGENCE OF UNINFECTED VALLEY PEA SEED AFTER TREATMENT WITH ANTIBIOTIC XG AND THEN WITH SPERGON, UNDER FIELD CONDITIONS

Seed treatment	Emergence, %
A. Unsoaked; with Spergon	71.0
B. Unsoaked; Spergon treated	83.0
C. Soaked in water; without Spergon	63.5
D. Soaked in water; Spergon treated	77.0
E. XG, 25 p.p.m., 18 hr.; without Spergon	70.0
F. XG, 25 p.p.m., 18 hr.; Spergon treated	72.0
G. XG, 50 p.p.m., 18 hr.; without Spergon	60.8
H. XG, 50 p.p.m., 18 hr.; Spergon treated	63.8

Least significant difference, 5.1.

Table X shows that Spergon treatment significantly increased emergence of unsoaked and soaked seed but not seed treated with Antibiotic XG. Spergon treatment of seed previously treated with Antibiotic XG, however, did not decrease emergence. Treating the seed with Antibiotic XG for 18 hr. with a concentration of 25 p.p.m. without Spergon treatment resulted in an emergence of the same order as the unsoaked check and significantly higher than in the case of the seed soaked in water. Antibiotic XG concentration of 50 p.p.m. significantly lowered emergence.

Discussion

On the basis of the limited laboratory and field tests reported in this paper, Antibiotic XG appears to be a promising disinfectant for pea seed infected with *Ascochyta Pisi*. There can be little doubt of its ability to inhibit the growth of the organism within the seed; and, in seed soaked for 18 hr. in a concentration of 25 p.p.m., it would appear to accomplish this with little detrimental effect upon seed germination. In the plate tests, a few seeds after treatment yielded *A. Pisi*, but these seeds failed to germinate. Under field conditions, such seed would probably fail to produce seedlings and, therefore, would not constitute a source of secondary infection. Disease control under field conditions could not be determined because of lack of isolation from infected plants. Antibiotic XG is compatible with at least one commercial seed protectant, namely, Spergon.

From the practical standpoint, Antibiotic XG has many favorable properties. It is stable for a period of eight months in acetone and for at least two months in alcohol (13); it is not highly toxic to seed at concentrations of 25 p.p.m., and its fungistatic action is not seriously affected by the pH of the

solution. A treatment that involves the soaking and drying of seed is not particularly desirable, but, in the absence of any other, it can be used as a means of obtaining at least small quantities of disease-free seed. The treatment is undoubtedly less exacting and is less injurious to the seed than the hot water treatment. In view of the small quantity of Antibiotic XG required to give the recommended concentration, the cost could probably be brought into line with that of other seed treating materials.

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DETERMINATE GROWTH IN THE BLUEBERRY[1]

By Hugh P. Bell[2]

Abstract

Elongation of the vegetative branch of the blueberry ceases early in June owing to the death of both the apical meristem and the distal portion of the axis. Coincident with this is the development of a flowering branch primordium in the axil of the penultimate leaf. During July, the apical meristem of the flowering branch becomes inactive. It is either laterally displaced or it elongates as a minute unbranched columnar structure. Coincident with this inactivation, the proximal flower primordia develop florets in which all flower parts may be recognized, but the distal flower primordia, that is those adjacent to the inactivated apical meristem, are retarded in their development. The retarded distal flower primordia are developed in acropetal succession but the proximal flower primordia do not exhibit acropetal succession. All the stages are illustrated by line diagrams.

Introduction

Determinate growth may result from the formation of an apical flower primordium or it may result from the death of the apical meristem. The inflorescence of the apple is a familiar example of the former type and has been described by Black (3, pp. 526–527). The latter type, namely determinate growth resulting from death or inactivation of the growing tip, occurs twice each season in the common blueberry. This growth cycle for the blueberry is described below.

Materials and Methods

The species used was *Vaccinium angustifolium* Ait. var. *laevifolium* House. For four years, collections were made twice each week from April to September and every month during the dormant season. The collections for any one year were made from one location, but, during the four years, three very different sites were utilized. During the first year, buds were taken from plants in a dry woods; in the second year from near a bog; and during the third and fourth years from open dry ground. The material was killed in FAA (air being removed with a vacuum pump), embedded in Tissuemat, cut 8 or 10 μ thick on a rotary microtome, and stained in safranin and fast green. Especially refractory buds were embedded in celloidin and cut on a sliding microtome.

The only way in which the technique departed from standard methods was in the dissection of the buds. Special precautions were necessary here, because it is always difficult to get adequate paraffin penetration with buds of the Ericaceae. To overcome this difficulty, even the smallest scales were either removed or loosened. Ordinary needles were much too blunt for this purpose. So to remove the small scales without injuring the terminal or axillary meristem, the dissections were done under a dissecting microscope, the material

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was illuminated with a beam of intense light, and the needles used were the tungsten type described by Bell and Facey (1, p. 131). It was necessary to keep these needles sharp.

Gross Morphology

The growth cycle, from a macroscopic standpoint, will be described by starting with the condition at the end of the dormant season, namely about the end of April. Exact dates are not significant, as the time for each stage varied considerably from season to season, although, for any one season, it did not vary from location to location. At the time just mentioned, that is just before the growing season starts, the distal portion of a branch appears as depicted in Fig. 1. There may be two flower buds but, for simplification, a branch with only one is figured (f.b., Fig. 1). This flower bud, when there is only one, appears to be terminal and is recognized by its large size and its shape, which is almost spherical. If there is a second flower bud, it is always the one next to the apparently terminal bud. Below this flower bud or these flower buds, there are varying numbers (usually from four to six) of vegetative buds. These are narrow in proportion to their length and are pointed at the tip. At the base of this branch is a withered twig (w.t., Fig. 1) which, as a flowering branch, bore the fruit of the preceding season.

Growth usually starts about the first week in May. Shortly after this, the flower buds burst open and the plants are in full bloom by the third or fourth week. While the flowers are emerging, the vegetative buds grow into vegetative branches. These attain almost their full length by the time the flowers are in full bloom. This length varies from one to four or more inches, the average length being about three inches and each produces, in succession, a varying number of bright green leaves. At this time, the axillary bud primordia are not conspicuous. This stage is illustrated in Fig. 2, which represents growth distal to line $x-----y$ in Fig. 1. The vegetative branch in Fig. 2 has developed from the distal vegetative bud (v.b.) in Fig. 1 and the flower cluster in Fig. 2, from the flower bud (f.b.) in Fig. 1.

The first indication of the determinate growth habit is evident during the last week in May or the first week in June, when a small black speck appears

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FIGS. 1 TO 4. *Diagrams for the blueberry representing the distal portion of a branch at significant stages.* FIG. 1. *The dormant season.* FIGS. 2 TO 4. *The growth line distal to line x---y in Fig. 1. Fig. 2, early May; Fig. 3, early June; Fig. 4, early August.*

FIGS. 5 TO 9. *Camera lucida outline diagrams of the branch apex and flowering branch. Magnification for Figs. 5 to 8, ×32; for Fig. 9, ×68.* FIG. 5. *Longitudinal section, apex vegetative branch, May 26; the withered tissue is stippled.* FIG. 6. *Tip of flowering branch, July 8, with the apical tissue somewhat displaced laterally.* FIG. 7. *Tip of flowering branch, Aug 7, with the apical tissue very much displaced laterally.* FIG. 8. *Tip of flowering branch, July 14, with an apical extension of the axis.* FIG. 9. *Composite diagram of a flowering branch, July 6, the flower primordia are numbered basipetally.*

For all the figures:— a. = apical tissue; c.t. = distal cataphyll primordium; e.x. = apical extension of the axis; f.b. = flower bud; f.b.p. = flowering branch primordium; f.p. = flower primordium; v.b. = vegetative bud; w.l. = withered leaf; w.t. = withered twig.

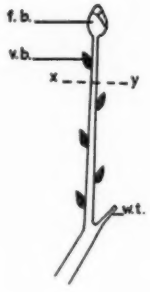

FIG. 1

FIG. 2

FIG. 3

FIG. 4

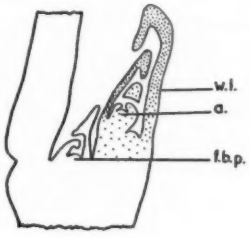

FIG. 5

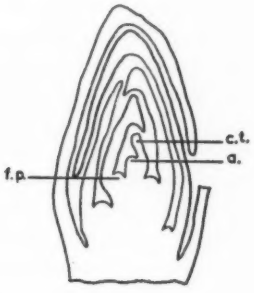

FIG. 6

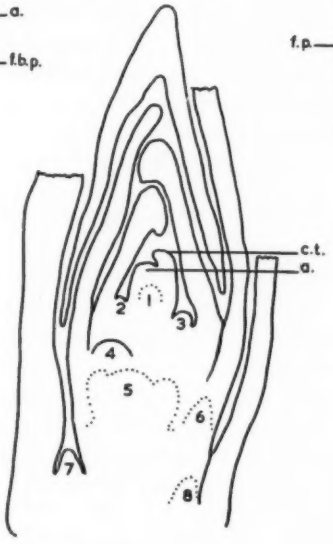

FIG. 9

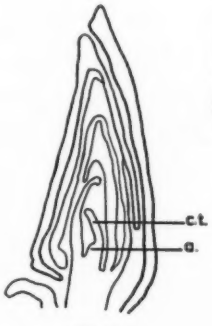

FIG. 7

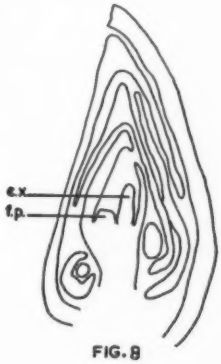

FIG. 8

at the tip of the vegetative branch (w.l., Fig. 3). On close examination, this can be recognized as the most distal and last formed leaf, dried and withered. Concurrently with this withering of the distal leaf, the axillary bud primordia along the vegetative branch enlarge, so that they may be seen quite easily. Also during the same period the corolla withers and falls off, leaving the ovaries (fruit for the current season) small and green but somewhat swollen. This stage is represented in Fig. 3.

Noticeable differentiation of the axillary buds along the vegetative branch occurs early in June. The withered tissue at the tip of the branch remains attached for a period varying from 10 days to two weeks, but by the end of the first two weeks in June, it has usually dropped off. From then on, the axillary primordium in the axil of the penultimate leaf enlarges and can be recognized as a flower bud. It enlarges so rapidly that the scar of the dehisced apex is soon crowded to one side and is finally obliterated. This flower bud then occupies its apparent terminal position. Another flower bud may or may not be produced in the axil of the antepenultimate leaf. As the season advances, the flower bud or buds continue to enlarge and assume the typical large size and almost spherical shape. The other and more proximal buds, which are usually vegetative, enlarge but become long and pointed. During the same period, the fruit matures. This stage is depicted in Fig. 4, which represents a branch during the last week in July or the first week in August, when the fruit is fully ripe. After the first or second week in August, when the fruit has ripened and fallen off, the branch that bore the fruit cluster withers and may be seen as a slender brittle twig at the base of the distal vegetative branch. By autumn, when the leaves have fallen, the whole structure is similar to that depicted in Fig. 1.

Histology of Distal Vegetative Branch Apex

For this part of the investigation only the distal vegetative branch was used because it could be relied upon to develop a flower bud. During the early stages of development, the apical meristem of this vegetative branch is not in any way atypical. During the initial elongation, when the leaves are being formed, this growing tip is decidedly convex, the cells are normal meristematic cells, and there appears to be one tunica layer. About the last week in May, that is, about the time that the distal leaf withers, two changes occur concurrently. First the primordium (f.b.p., Fig. 5) in the axil of the penultimate leaf enlarges considerably, and second, the meristematic cells of the branch apex (a., Fig. 5) become vacuolated and inactive. The distal portion of the youngest leaf (w.l., Fig. 5) has withered; hence it stains deeply. This withering may be traced as it progresses basipetally from the tip of the youngest leaf, reaches the axillary meristem at its base, and then includes the portion of the axis distal to the primordium developing in the axil of the penultimate leaf. The withered tissue forms the black speck referred to in the description of the gross morphology and in Fig. 5 it is stippled. All this withered tissue dehisces

and then the primordium in the axil of the penultimate leaf (f.b.p., Fig. 5) develops a flower branch primordium which, histologically as well as in gross structure, appears to be terminal in position.

Histology of the Flowering Branch

Preliminary Statement

For the study of the flowering branch, the distal and apparently terminal bud of the distal vegetative branch (f.b., Figs. 1 and 4) was collected exclusively, for it could be relied upon to develop a flowering branch. This flowering branch will be described under three headings, namely:— (*a*). The Apex; (*b*). Flower Primordia, Early Stages, and (*c*). Flower Primordia, Later Stages.

(*a*). *The Apex*

Through June and the first week in July, the flowering branch elongates and broadens within the bud and this is accompanied by the enlargement of the bud mentioned in the description of gross morphology. During the first of this growth, that is, approximately throughout June, the apex of the flowering branch (a., Fig. 9) is a normal apical meristem, but, by the second week in July, the meristematic cells of the apex become vacuolated and inactive. The distal cataphyll primordium (c.t., Figs. 6, 7, and 9), however, continues to enlarge in all dimensions. As a result, the apex is displaced to a lateral position (a., Fig. 6) and, by August, it is an insignificant mound of tissue lateral to the base of the most distal and smallest bud scale (a., Fig. 7). In the study of such an apical meristem, it is very easy to make an incorrect interpretation of its position, for as pointed out by Bell and McLellan (2, p. 341), sections of exactly similar apices may have very different appearances according to the plane in which they are cut. With this in mind, many sections were checked with the greatest of care and there appeared to be no doubt regarding the lateral displacement of the branch apex, but the amount of displacement varied from bud to bud. Finally, with the growth of all other parts, this apex may become so comparatively insignificant, that it is difficult and at times impossible to identify it with certainty.

The condition just described and illustrated is the usual determinate growth habit in the flowering branch, but in a certain number of plants examined (roughly about one in from four to six), the apex continues to elongate after the formation of the distal axial flower primordium. This elongation results in the formation of a cylindrical structure (e.x., Fig. 8) with a rounded tip and composed of vacuolated cells. It may be from 145 to 160 μ high, is circular in cross section throughout the whole length, is 29 μ in diameter near the tip, and 73 μ in diameter at the base. It is unbranched. To a Maritimer, this extension of the axis looks like a microscopic lighthouse without a light. In a freshly dissected bud, its gross appearance is like a fine, straight, yellowish-green hair. At first, it was regarded as a bract or epidermal hair of some type,

but careful examination of serial sections, both longitudinal and transverse, revealed unmistakably that it is an elongation of the branch axis. No explanation is offered for this structure or its development. It is illustrated in Fig. 8.

(*b*). *Flower Primordia, Early Stages*

While the flowering branch is enlarging, that is during June and early July, from 7 to 10 flower primordia develop in the axils of the cataphylls. The primordia of these cataphylls were developed acropetally, but this succession is not obvious in the development of all the flower primordia, for the enlargement of the proximal four to six occurs at such an early stage and so rapidly that a succession could not be determined. That is, at any one time after they could be definitely recognized, all these proximal primordia appear to be at about the same stage. This is not the case with the three or four distal flower primordia, for, with them, an acropetal development is obvious. These facts can be brought out by examining one branch in detail. This can be done by reference to Fig. 9, which represents diagrammatically a bud collected on July 10. It is typical for all buds collected about that date. Fig. 9 was constructed from a series of longitudinal sections. In this bud, the apical meristem was becoming vacuolated but its position was still strictly terminal. There are eight flower primordia and, for reference, they are numbered basipetally. That is, No. 1 is the primordium nearest the apex (a). Primordia Nos. 1 and 6 are above and Nos. 3 and 8 are below the central plane. All these primordia are flattened radially and elongated tangentially. If viewed in transverse section, they would be crescent-shaped. Their respective sizes can be compared by considering the tangential measurements. For this bud, these measurements expressed in microns are as follows:— No. 1, 51; No. 2, 64; No. 3, 120; No. 4, 128; No. 5, 153; No. 6, 160; No. 7, 200; No. 8, 140. Respecting the development of these primordia, Nos. 1 and 2 are just mounds of meristematic tissue. The first indication of floral parts may be detected in No. 3, but in Nos. 4 to 8 development has proceeded much further. All the evidence indicating these stages of primordium development could not be included in Fig. 9 and additional information was obtained from both longitudinal and transverse series through other buds of the same period. The tangential measurements suggest that Nos. 1 to 7 are in acropetal succession and that No. 8 is not so, but to use size alone for determining the succession of development would be deceptive, for, judging by extent of flower-part formation, Nos. 4 to 8 are all at about the same stage, and, although No. 8 is the smallest of these proximal primordia, it is as advanced as any. Hence, taking all available evidence into consideration, as yet, it cannot be stated that acropetal succession is followed completely in the development of the blueberry inflorescence.

If the blueberry is compared with other plants, the tangential measurements suggest that primordia Nos. 1 to 7 are like the basipetally located flower primordia described by Bell and McLellan (2, pp. 352–353) for the apple, the main difference being that, in the apple, determinate growth is inaugurated

by the apical meristem developing a flower primordium, whereas, in the blueberry, the apical meristem becomes inactivated. Perhaps the inflorescence of the blueberry is more like that of *Garrya* as described by Reeve, where there is "a correlation of the abortion of the upper florets with the retardation and eventual loss of meristematic activity in the apical portion of the axis." (4, p. 611). However, before comparisons with other plants would be of much value, additional details covering such subjects as vascularization, etc. should be obtained for the blueberry.

(*c*). *Flower Primordia, Later Stages*

During the remainder of the growing season, that is from the middle of July on, the flower primordia continue to enlarge and differentiate, till by the autumn, at the start of the dormant season, the stages reached vary somewhat from bud to bud but in some cases they are as follows:— No. 1 has not developed beyond the axillary primordium stage; Nos. 2 and 3 form distinct flower primordia, exhibiting the first indication of flower-part formation, No. 3 being slightly more advanced than No. 2. In contrast to these distal primordia, all the flower primordia basipetal to No. 3 are completely differentiated florets in which all flower parts may be recognized. That is, the five or seven basipetally placed flower primordia are similar, for they enter the dormant season at the same advanced stage, whereas the three distally placed primordia are not only much less advanced but considering the stage and size of each, they are in acropetal succession. The basipetal flower primordia would form the flowers of the succeeding spring, but the ultimate fate of the branch apex and the distal flower primordia was not determined. However, at blossom time, the flowering branch in each flower cluster is terminated by a short crooked, withered twig. It is possible that this twig may be the portion of the flowering branch distal to primordium No. 3 or No. 4.

Discussion

If one considers the facts presented above from the standpoint of their possible relation to growth substances, inhibitors, growth gradients, etc., obvious theoretical deductions might be drawn from these manifestations of the determinate growth habit, but owing to the variations in these manifestations, the deductions might be various and even contradictory. For instance, the determinate growth of the vegetative branch is inaugurated by the death of the apical meristem and is accompanied by rapid development of the axial meristem nearest to this dead or dying tissue. In the flowering branch, determinate growth is inaugurated sometimes by the inactivation and lateral displacement, but not death, of the apical meristem and at other times by elongation of the apex. In either case the change in the apex is accompanied by inhibition of activity in the nearest axial meristem and the rapid development of the more distant basipetally located meristems. However, in both

branches, the inauguration of the determinate growth habit is correlated with the initiation of axial primordia. For the present, no theoretical implications are suggested, but the facts appear to be worthy of record.

Acknowledgments

This work was carried out with the technical assistance of Miss Laura J. Bisset and Miss Jane N. Bailly. Miss Anne L. Gorham drew all the figures.

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STUDIES IN *SEPTORIA LYCOPERSICI* SPEG.[1]

By Blair H. MacNeill[2]

Abstract

Investigations into the biology of *Septoria lycopersici* Speg., a leaf-spotting fungus parasite of the tomato, reveal that this fungus species is composed of at least two physiologic races which show both qualitative and quantitative differences in their pathogenicity. Other factors, such as humidity, temperature, light, and host nutrition, which influence host–parasite interaction, have been shown to cause further variability in the symptomatological picture. Studies of spore germination and the prepenetration phase of host–parasite relationships suggest an interaction before actual penetration which, in some cases, is sufficient to condition the germination of the spores themselves. A minimum period of 48 hr. at saturation is required to promote germination and stomatal penetration, but this experience at high humidity need not be continuous. The fungus inhabits an intercellular locus, but is very intimately associated with the cells of the tissues parasitized. There is no evidence of action in advance; rather a certain measure of host–parasite equilibrium is maintained, and the pathogen is enabled to complete its life cycle and reinitiate infection. Although this evidence suggests a rather high degree of specialization of parasitism, the nature of the host–parasite association is yet considerably removed from that of an obligate relationship. It is pointed out that while *S. lycopersici* might be considered, in the sense of de Bary, either as a facultative saprophyte or an obligate parasite, its peculiar behavior makes it exceedingly difficult, if not impossible, to place it into any one category which might describe its biological activity.

Introduction

The present studies were undertaken in order to determine some of the fundamental aspects underlying the parasitic activity of *Septoria lycopersici*. This fungus occurs with persistent regularity in the field tomato crop of Ontario, and has been the object of considerable research dealing with its eradication or practical control. Yet, a review of the literature reveals that little is known about those aspects of a pathology which must deal with such problems as physiologic races, predispositional factors of environment and nutrition, and host–parasite interaction; accordingly, it is hoped that this study of *S. lycopersici* will help to elucidate some of these problems, as well as to throw some light on the parasitism of a group, the pycnidiaceous fungi, which, thus far, has been but little investigated.

It was partly in appreciation of the foregoing, and partly in view of the fact that *Septoria* leaf spot is a very serious disease in Ontario, that the tomato breeding program at the Vineland Horticultural Experiment Station was organized to promote these studies, and the facilities of the Station made available to us for the carrying out of the greater part of the work herein reported.

[1] *Manuscript received September 8, 1950.*

This paper constitutes part of a thesis submitted to the Graduate School of the University of Toronto in conformity with the requirements for the degree of Doctor of Philosophy. Part of the work recorded here was carried out as a co-operative project between the Department of Botany at the University of Toronto and the Horticultural Experiment Station at Vineland.

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Symptomatology

The first visible evidence of leaf infection is a slight chlorosis most easily distinguishable in transmitted light. This chlorosis becomes a halo around the lesion which develops soon after, while the lesion is marked by the collapse of the host tissue earliest colonized. Under optimum conditions a period of four to six days is necessary for the fungus to enter the host and establish itself to the extent of producing such symptoms, while adverse conditions, especially of moisture, may lengthen this period but never shorten it.

The ordinary susceptible spots are from 2 to 4 mm. in diameter, and by the end of the 10th day, or earlier if the atmosphere has not been allowed to drop too low in moisture content, scattered pycnidia become evident in the central portion of the collapsed areas. Under field conditions, where fluctuating conditions of moisture and temperature exist over extended periods, the spots may increase from two to three times their original size, though their capacity to sporulate appears not to be proportionately increased.

Liberation of spores begins soon after the appearance of the pycnidia on the exterior of the leaf. Secondary infections from oozing pycnospores may involve nearly the whole leaf under conditions of high moisture.

The development of the disease in young plants is rather strictly confined to the lower half of the plant on the more mature leaves, the younger leaves remaining conspicuously healthy. Under field conditions it seems probable that the expanding leaves become infected from exuding pycnidia on the older leaves which received the original inoculum. Defoliation is considerably hastened by infection. In older fruiting plants this gradient of susceptibility is somewhat displaced, and the senescing leaves at the bottom are no longer readily attacked; the upper half of the plant, however, still exhibits a difference in degree of susceptibility as between the older and the younger leaves.

The disease is almost strictly foliar though under certain conditions lesions may appear on petioles, stem, and the calyx surrounding the maturing fruit. Such spots rarely, if ever, produce spores, although pycnidia may develop in the calyx spots.

Physiologic Studies of the Causal Organism

Effect of Temperature

At the outset, germination tests were run to determine the amount of variation due to age of the spores used. The best germination was secured in spores freshly exuded from pycnidia, while a matter of only a few days difference in the age of spores gave results that were very inconsistent regardless of the temperature of the medium.

In hanging drops or on solid media spore germination occurs within 48 hr. over a wide range of temperatures, with an optimum in such artificial cultures falling between 27° and 29° C. Consistent and vigorous germination occurs

at a temperature as low as 18° C., but there is a rapid falling off of the temperature curve on the upper side of the optimum with no germination at all at 31° C.

Plasticity in Culture

Regulatory Effect of the Medium

In addition to the stock types of media, potato dextrose agar (P.D.A.) and tomato leaf extract, several other sources of nutrient were incorporated in a series to determine the influence of the substrate on the developmental rhythm of *Septoria*. Was there a regular fruiting period of, say, eight days, or could this be controlled and regulated by the substrate? (Cf. plasticity of reaction on hosts under different environmental conditions of light, nutrition, humidity, and temperature.)

The following nutrient sources were used: tomato leaf extract, tomato fruit extract, sweet cherry juice, sour cherry juice, strawberry fruit extract, and extract from tomato roots.

Fruit extracts were prepared by preheating the fruit at 135° F., extracting the juice while still hot, and then pasteurizing it at 180° F. The extracts from leaves and roots were obtained by grinding the raw material, extracting it through cheesecloth, and autoclaving it to sterilize. Test tube slants were then made by adding, in separate series, drops of an extract such as to give a range of concentration of the medium of from approximately 0.01% to 50%. Checks were kept on both plain agar, and liquid culture of the extract being tested, as well as on P.D.A. which was used as a standard medium with which all others were compared. Cultures were incubated at room temperature with duplicates at 25° C.

The results with the extracts used showed that the response of the fungus to the dilutions was one that followed a definite pattern with respect to vegetative growth and the fruiting cycle. High concentrations produced heavy vegetative growth with considerable aerial mycelium and good pycnidial formation. Very high concentrations of extract often caused the formation of abortive pycnidia. Lowering the concentration of the extract reduced the vegetative growth but did not affect the amount of fruiting proportionately. Since similar responses were obtained with sucrose and dextrose, it is believed that the main regulatory constituent in this respect is the carbohydrate contained within the medium. The most effective concentration of sugar for good pycnidial formation was between 0.33 and 1.33%; at such concentrations there was a minimum of vegetative growth and an abundance of sporulating pycnidia. It should be noted, however, that germination and the eventual production of spores occur on plain agar to which no carbohydrate is added; on such a medium there is little growth other than the fruiting bodies themselves, and even they are by no means abundant.

While the various media used produced overall responses that followed definitely similar trends with respect to vegetative growth and pycnidial production, there were differential effects upon the periodicity of fruiting in

Septoria. In one series, P.D.A. produced sporulating pycnidia in seven days; in the same series 3% dextrose agar displaced the development of pycnidia and the oozing of spores to a nine day period. Sweet cherry extract agar, while promoting eventually a heavy production of spores at concentrations of 0.5% to 1.5%, did not allow for the completion of the fruiting cycle in fewer than 10 days, and a maximum production of spores was not reached until the 12th day. Similarly, maximum fruiting did not occur on green tomato fruit extract until as late as the 17th day. Good pycnidial formation occurred on tomato root extract agar at the end of eight days.

In none of these cases, providing the pH was favorable for germination and subsequent growth, did the reaction of the medium appear to affect the periodicity of pycnidial formation or spore production.

From this series of experiments it is apparent that while one may postulate a regularity in the fruiting cycle as it is known from the habit of the fungus on a specific medium, there is yet a plasticity in this cycle that is regulated, to some extent at least, by the substrate upon which the organism is being cultured. The evidence at hand indicates that such a regulatory effect of the medium may displace this developmental rhythm to a considerable extent, and even in some cases prolong that period required before spore production is at a maximum, to almost twice that ordinarily looked upon as normal.

Nutrition of the Fungus in Culture

A range of carbon sources including mono-, di-, tri-, and polysaccharides were incorporated separately in a Czapek's mineral nutrient medium, and parallel experiments run, one of which was on slants of this medium hardened with agar, the other as a liquid medium distributed in hanging drops. Inoculated tubes and drops were incubated at 25° C. Czapek's without added carbon sources served as checks.

The carbon sources tested were glucose, galactose, levulose, arabinose, xylose, rhamnose; maltose, sucrose, lactose; raffinose and soluble starch. In addition, strips of sterile filter paper standing in Czapek's solution in tubes were used to determine the availability of carbon in cellulose.

Best growth and sporulation occurred on glucose, although there was little to choose between the behavior here and that found on sucrose. An almost equal productivity was attained on the pentoses, arabinose, and xylose. Galactose, rhamnose, maltose, and raffinose supported good vegetative development but spores were produced meagerly if at all. Little vegetative growth occurred on either lactose or levulose, although the latter promoted fruiting. Soluble starch did not support growth, and similarly the carbon in the filter strips seemed to be unavailable.

Combinations of two or more of the above carbon sources did not serve to stimulate fungal development beyond that occurring with the best of the carbon members when it was used alone.

The above observations would seem to suggest that the character of the development of *S. lycopersici* in artificial culture is, to a large extent, a function of the availability of carbon in the substrate, and that vegetative activity and reproduction may be regulated independently of each other by separate factors of nutrition.

Effect of Humidity in Artificial Culture

The open faces of the covers of two Petri dishes were placed together and the edges cemented with vaseline. The lower half contained a sulphuric acid solution of known concentration while the upper inverted half held solidified P.D.A. The acid solutions were made up according to the method of Stevens (11) and calculated to give a range of relative humidities within the series of containers. The agar was thus exposed to an atmosphere of known humidity as regulated by the concentration of the acid solution over which it was suspended. A period of 12 days was given to allow for the agar to lose moisture and to come to equilibrium with the atmosphere within the closed container. It was appreciated that the absolute values of relative humidity (R.H.) as given by Stevens for the acid series could not be accepted for the above experimental setup, but that one could get at least a comparative R.H. series. In each case, owing to water introduced with the agar, the R.H. within the container would be as high, if not higher, than that expected from the acid concentration used.

At the end of the 12-day period noted above, the agar surface was inoculated with a drop of spore suspension which was very thinly smeared over the surface of the substrate. The vaseline seal was renewed and the containers incubated at 25° C.

Germination and vegetative development occurred by the end of the fifth day at all humidities down to and including an initial R.H. of 96.2%. At the lower end of the range of humidities, however, there was a high percentage of nongerminated spores. Plates which showed no germination at R.H. as low as 95.6% showed germination and vegetative development when distilled water was added to replace the acid and so give a saturated atmosphere within the container. Spores which had been exposed for 10 days to relative humidities lower than 94.6% did not recover.

This experiment was repeated but modified to the extent of using a graded series of sucrose solutions, according to the method of Shaw (10), instead of sulphuric acid to produce the desired humidities. In this series germination occurred at R.H. as low as 96%. The differences in results are considered to be acceptable within the limits of experimental error.

It will be noted that the results here correspond rather closely with those obtained with inoculations of plants maintained under constant conditions of relative humidity. While some flecks were found to occur at 85% R.H., no symptoms that could be attributed to effects of the fungus itself were ever

found on inoculated plants maintained at an R.H. of less than 97%. These observations would seem to support Yarwood's thesis (13) that the humidity at leaf surfaces approaches quite nearly that of the surrounding atmosphere.

Stimulation of Spore Germination by Exosmosed Foliage Products

In ordinary tap water and in distilled water, germination of spores is at best very weak; accordingly, it was of interest to determine the cause of the seemingly paradoxical circumstance whereby germination proceeded vigorously when the inoculum of water–spore suspension was applied to the foliage of the living plant.

To test for this factor, small glass rings were cemented to the surface of the leaves of a tomato and a few drops of distilled water placed within these glass reservoirs. A cover slip was fastened over the top of this cell to keep out dust particles and condensed moisture. After a period of 48 hr. the liquid was drawn off from these cells and tested against freshly distilled water for its capacity to induce consistent germination. It was found that such liquid which had been in contact with the leaf surface for 48 hr. now acted as a favorable medium for spore germination, and that while no vegetative growth of the developing germ tubes took place, the consistency with which germination resulted was significantly higher than that of the checks. It is suggested then, that some product of host metabolism was "exosmosed" into the glass reservoirs and that this acted to stimulate spore germination in the artificial culture. Accordingly, it is further suggested that such an exosmosis occurs into the inoculation drop when the plants are naturally infected or artificially inoculated with spore suspension, and that this interaction between host and parasite prior to the penetration phase is important in conditioning the character of germination, whether it be to stimulate this process or to inhibit it. In this connection it is to be noted here that the extent to which the effect of exosmosis is operative in differentiating resistant and susceptible hosts is not clear from these experiments, since the same stimulation of spores was observed in the drops taken from the foliage of plants of the resistant *Lycopersicon hirsutum* Humb. and Bonpl.

Pursuing further the above observations, it was noted that fortifying a water–spore suspension with such nutrients as dextrose, peptone, and asparagine did not, apparently, expedite the process of parasitizing a susceptible host such as Bonny Best tomato in any noticeable fashion.

Host–Parasite Relationships

Penetration

Entrance into the host is entirely stomatal; no evidence of direct penetration was ever noted. Spores which have germinated on the surface of the leaf may penetrate after the production of a very short germ tube, and this is often the case when the spore happens to lie across a guard cell; however, more often there is considerable wandering of the germ tubes and the developing vegetative

branches before a stomate is actually entered. It is not unusual to note an open stomate across which the fungus has grown without any apparent attempt to gain entrance through it. On the other hand, penetration may be accomplished through the first stomate contacted by the germinating spore (Fig. 3).

Studies upon the character of penetration of compatible hosts were made with the susceptible Bonny Best tomato. Inoculated leaves which had been sprayed with a water–spore suspension, or dotted with drops of the suspension and the areas marked with small bits of cotton, were sampled at intervals. These leaves were excised and dropped immediately into a warm solution consisting of equal parts of glacial acetic acid and 95% ethyl alcohol. This solution gave reasonably good fixation, extracted the chlorophyll, and cleared the leaf generally, though the chief reason for its being used was that it gave good fixation of the germinating spores, and helped retain them in position on the leaf surface. Clearing the leaves in alcohol dislodged the spores that had not already anchored themselves by their penetration tubes in the mouths of stomates. The leaves thus treated were mounted on a slide and stained with lactophenol – cotton blue, and examined microscopically.

While penetration may be accomplished by the entrance of what appears to be merely an extension of the growing germ tube which proceeds readily between the guard cells, more often it is characterized by an appressorium-like enlargement which comes to lie immediately in the depression which delimits the outer mouth of the stomate. From this enlargement a further development of a tube, much reduced in diameter, occurs; it is this penetration tube that negotiates the remaining distance to the substomatal chamber. It is not uncommon to find hyphal development occurring at the same time from the aforementioned appressorial enlargement, even though the stomate has been successfully penetrated. This new growth may be directed along the leaf surface and effect penetration of a stomate some distance removed. No penetrations through other than open stomates have been observed.

No immediate reaction of the host is apparent during these early phases of host–parasite contact. From the substomatal chamber, in which considerable enlargement of the fungus now takes place, runner hyphae proceeded in all directions. These hyphae maintain a subepidermal locus for a horizontal distance of several cells beyond the substomatal cavity, although if penetration has been on the upper leaf surface these hyphae proceed almost immediately down between the palisade cells.

The mechanism of penetration of incompatible hosts is the same as that described for that of the compatible hosts. An appressorium-like structure is formed in the mouth of the stomate, and from this a penetration tube subsequently grows through the stomatal aperture. While the amount of colonization that follows successful penetration is, in part, an indication of the compatibility of host and parasite, the path followed by the invading hyphae is essentially the same as that described for the compatible host.

Host–Parasite Interaction

Investigations of the tissue relationship of host and parasite were carried out with the aid of hand sections made with a razor. Fresh leaf material was used after clearing with acetic–alcohol mixture, and infected areas delimited and sectioned in dry pith. Lactophenol–cotton blue proved to be a very satisfactory stain for the material under study; sections were simply mounted in a drop of this medium, and examined immediately under the microscope. Microchemical tests were made upon fresh living sections as well as on those killed and cleared as above.

As noted earlier, there is not any readily apparent reaction on the part of the compatible host to the invading fungus during the initial stages of colonization. The mechanism of parasitism, then, is not one of action in advance, although in the later stages of colonization there is some host response in regions not in contact with the parasite.

The locus of development of the fungus in the living tissues is entirely intercellular; however, advancing mycelial strands become closely appressed to the cells of the palisade and spongy mesophyll tissues, and the contours of the cell walls are reflected in the character of the mycelium as a result of this intimate association between host and parasite. An examination of razor sections of fresh material reveals the twisted, almost tortuous, path of the hyphae as they develop in the rather confined intercellular spaces and along the surfaces of the cell walls. In sections cut obliquely, or where there is little depth of focus under the microscope and the true association of fungus and host cannot be observed, the bulbous nature of the hyphal strands as they abut the cell walls suggests haustorial development. Microtome sections cut from embedded material likewise may be misinterpreted, as is revealed when one obtains good sections of fresh material; there is no penetration of the cell walls in this parasitic association of the host and fungus, and the evidence at hand does not support that offered by Levin (7) and Harris (5) who reported the presence of haustoria as suggested from their examination of embedded materials. We have found that it is only in such tissues where there has been some degradation of the cellular constituents that the fungus can, in any sense, be considered intracellular, and then it is in dead, not living tissue.

Those tissues earliest colonized seem to be the first to succumb. The first manifestation of this reaction is the plasmolysis of the cells; this is quite readily discernible in the plastid bearing tissue where the clumping of the chloroplasts and their eventual disintegration is a notable feature of the parasitic attack. Histologic examination of infected areas shows the complete collapse of the epidermal cells under which the runner hyphae had previously progressed, while cells of the palisade and spongy mesophyll show the almost complete breakdown of the contents as noted above, and often a shrinking of the cell walls; in extreme cases there may be some breakdown of the cell walls of these tissues.

In those regions where the palisade or other mesophyll tissues are in such close arrangement with the epidermal cells that the invading fungus is strictly confined to limited intercellular spaces during this early colonization of the subepidermal locus, there seems to be considerable attack upon the pectic elements. Sections revealing the fungus and host in such an association give either a very faint test or a completely negative test with ruthenium red. Plasmolysis of both the epidermal and plastid bearing cells concerned follows this attack. The chloro–zinc–iodide test for cellulose is not seriously interfered with, and there seems to be no real attack on the cellulose elements at this time.

The earliest indications of pycnidial formation appear at about the fourth day of incubation, by which time the knots of fungus developing in the spongy tissue are quite readily apparent. A study of the mode of pycnidial formation and spore development has been made by Harris (5) and the evidence, also observed here, would indicate that the pycnidial primordia arise through an aggregation of several hyphae that intersect in the leaf tissue and intertwine to form a conglobate mass of hyphal tissue. The extrusion drop accompanying the spores through the ostiole is said by Harris to result from the gelatinization of hyphal elements within the mature pycnidium.

A rather conspicuous feature of the later stages of pycnidial formation is the appearance of a brown discoloration in the infected tissues, and in some cases in those tissues not actually colonized. Often this browning reaction may extend for a considerable distance along the veins although these tracheal elements may not be invaded by mycelium. It is appreciated that this may be nothing more than a wound reaction, although the unilateral advance of this browning into tissues remote from the actual hyphal colony would suggest some toxic product derived from the host–parasite interaction.

In these investigations it was observed further that the greater part of the protoplasmic content of the colony was expended in the production of the fruiting body and its huge quantity of spores. Histologic examination of infected regions reveals that the outermost reaches of the colony no longer take the cotton blue stain, and apparently the protoplasmic contents have retreated from the hyphal tips and moved toward the fruiting body. The fact that it is difficult to culture the fungus from infected tissue remote from the sporulating fruiting body would indicate that the vitality of the fungus, once spores have been produced, is of an extremely low order. It is suggested that this may, in a large part, account for the rather restricted spread of *Septoria* leaf spots.

As part of the researches to be discussed later in connection with the search for differential host materials, infection studies were carried out on a range of hosts other than the susceptible *L. esculentum* (Mill.) varieties.

On the resistant host *L. hirsutum*, penetration was found to be stomatal and to occur with sufficient frequency to bring about a serious leaf-spotting should the infection develop into a general colonization of the tissues. However, no

such development occurs, and a rather violent reaction by the cells immediately adjacent to the penetration zone apparently restricts the fungus to its stomatal locus. These cells react with the production of an intense pigmentation accompanied by the plasmolysis and death of the protoplast. It has not been found possible to recover the fungus in culture from such lesions on *L. hirsutum*, although on the susceptible esculentum-types the fungus can be recovered as soon as 24 hr. after actual penetration, and at any time subsequent to this.

The potato, *Solanum tuberosum* L., was found to be readily infected by *S. lycopersici*, and microscopic examination of inoculated leaves of several varieties of potato indicates that germination and penetration occur as readily as on the compatible *L. esculentum*. Such infections do not, however, complete the cycle and produce a diseased condition with sporulating lesions; instead, there is a pigmentation of the cells within the locus of infection, and a general host reaction which would indicate considerable irritation as caused by the parasite. While there is this rather sharp interaction between host and parasite, it is not such as to destroy the invader completely, as happens in infections of *L. hirsutum*; rather, the fungus can be recovered some full three weeks after infection, even though the fungus has never been able to organize a spore-producing body within the potato leaf tissue. Evidently, there is maintained within the host tissue an environment sufficiently favorable for the continued survival of the parasite.

The fact that this fungus in its nonsporulating cycle can be recovered after a considerable interval is in striking contrast with the condition noted earlier in which the protoplasmic contents of the colony evidently are expended in the production of spores, and the fungus as a consequence is irrecoverable from the lesion, except of course as a culture from the spores themselves.

Prepenetration Effects

It has also been of interest throughout this phase of these investigations to note what appears to be a definite prepenetration effect in the host–parasite relationships of *S. lycopersici* and certain plants with which this parasite visibly reacts.

The fact that there may be at least some interaction between host and parasite before actual penetration has been noted by Brown (2) through his experiments with exosmosis. A similar phenomenon has been observed here, although the paradoxical situation with respect to exosmosis effects on susceptible and nonsusceptible hosts would indicate the presence of other conditioning factors not demonstrated by our experiments.

And yet, in spore inoculations of *L. hirsutum* it is evident that while some spores germinate normally and penetrate through the stomates, there are many spores that do not germinate at all. This is to say that there is a considerable reduction in the frequency of spore germination as conditioned by the host upon which the spores come to lie. While we do not have actual counts of percentage of germination which could be subjected to statistical analysis, we

feel, nevertheless, that our familiarity with the biological material at hand sufficiently underwrites the data to permit us to postulate such a prepenetration effect. At the same time we note that the percentage of germination is even more drastically reduced on *Petunia*, which is immune. In all cases the susceptible Bonny Best was used as a check for comparison in these observations.

Influence of Environment on Disease

Predispositional Effects

Influence of Host Nutrition

Series of tomato plants, including Bonny Best and the resistant types SWST and S25, were grown under liquid nutrient culture in pots containing vermiculite as a rooting medium. Seeds were surface sterilized and sown directly into the vermiculite, and the pots were kept moist with plain tap water until the emergence of the cotyledons, after which time the seedlings were separated into groups for differential treatment with a range of liquid nutrients. These groups were designated by letters according to the type of nutrient received: R, 4R, R/4, N/4 PK, N P/4 K, NP K/4. The "R" (normal) solution was according to the formula of Waygood (12).

In the quadruple strength 4R the growth was luxuriant with dense green foliage, although the overall height was not much greater than that obtained with the normal solution R. The quarter strength, R/4, gave a stunted plant that early showed a chlorotic condition with a general, hardened appearance that foretold marked deficiency symptoms by the time the series was ready to inoculate. Reduction of phosphorus or potassium singly, to quarter strength, had the effect of producing plants that were slightly reduced in height but otherwise showed no foliar effects of the deficiency. On the other hand, reducing the concentration of nitrogen to one-quarter that of the R solution produced a marked stunting of the plants followed by a rather severe chlorotic condition. Thus, by the application of a mineral nutrient solution in which the constituents were varied within certain controlled limits, a differential physiologic condition was promoted during an interval which extended through the seedling stage of the tomato plants and carried on for several weeks into the later maturing stage. The result of this treatment was a series of tomato plants that showed marked differences in appearance as expressed in size, color of foliage, and general vigor.

These plants were sprayed with spore suspension and placed in an incubation chamber for 48 hr. Readings were taken on the seventh day, and the amount and type of infection recorded. Differences in susceptibility were noted in the plants that could be attributed only to the predisposition of the host through these differential nutrition treatments. Check plants (R group) showed heavy infection, with large spreading lesions on the susceptible Bonny Best, and an equally heavy attack on the leaves of the resistant varieties,

although here the resistant character, as usual, resulted in a typical, restricted type of lesion which has a considerably reduced capacity for sporulation. In the same way Group 4R became very heavily infected.

The series N P/4 K and NP K/4 gave infection pictures not unlike those which had received the balanced nutrient. The susceptible Bonny Best developed a heavy infection with abundant sporulation, while the resistant types SWST and S25 showed the typical resistant type lesions with the usual amount of sporulation occurring.

On the other hand series N/4 PK, in which the nitrogen alone had been reduced, presented a somewhat different picture. Here the amount and type of infection taking place in the resistant stocks was markedly less than that found under either a balanced nutrition or in any of the nutrient series used in which the concentration of nitrogen approached that found in the normal R solution. As was noted above, SWST and S25 under balanced nutrition express a certain degree of resistance to *Septoria*. This resistance is, for the most part, one of restricted lesion size and a resultant reduction in pycnidial loci; however, there seems not to be any marked resistance to attack by *Septoria*, for SWST, S25, and Bonny Best may become equally heavily infected, in so far as numbers of lesions are concerned, when all are maintained under optimum conditions during the incubation period. The reduction of available nitrogen serves but to emphasize the resistant character, and there is a consequent displacement toward an even more resistant reaction. Lesions go through only a very restricted development, sometimes being reduced almost to flecks, while the overall symptomatological picture is one of marked reduction in foliar infection and a resultant lessening of the amount of defoliation (Figs. 9 and 10).

A similar predisposition effect upon the resistant character was expressed under R/4 nutrition; it is presumed that here again this type of expression is due to the nitrogen level of host nutrition, since reduction of phosphorus and potassium separately had no visible influence on the amount and type of infection developed.

Such a displacement of the *Septoria* reaction is not occasioned by a reduction of the available nitrogen to plants that have no inherent resistant character. The susceptible Bonny Best contained in the above series gave a consistently susceptible reaction with large spreading lesions and abundant sporulating pycnidia, regardless of the nutrient available.

Effect of Light

The extreme sensitivity of the tomato plant to fluctuations in light experience makes it difficult to evaluate the effect of light upon disease expression. Low light intensities, such as those that prevail during the winter months in a Toronto greenhouse, produce seedlings with leaves that are thin, flaccid, and pale in color. Such extreme displacements in the physiology of the host tend

to distort disease symptoms, and inoculation results cannot, accordingly, be interpreted with any assurance of general applicability either as to type or plasticity of reaction.

Some indications of the effects of light were obtained, however, by taking healthy tomato plants as produced under summer conditions, and artificially regulating their light experience by shading them under cloth tents. Three degrees of light intensity were obtained in this way: full light, medium light under one layer of factory cotton, and weak light under one layer of black cloth. Both the susceptible Bonny Best and the resistant types S25 and SWST were included in the series. These conditions were maintained for three weeks, after which time the plants were inoculated and kept at saturation for 48 hr. At the end of 48 hr. the plants were returned to their original differential treatments. Reactions were read on the 7th and 14th day.

The effect of reduced light in all cases was to intensify the severity of disease. Even a few lesions per leaf caused very severe defoliation. Lesions were larger under the reduced intensity, and at low light became so spreading and diffuse that large areas of the leaf were affected.

Reducing the light from full to medium gave a more obviously differential reaction than did the further reduction of light from medium to low. However, plants under low light were rather seriously chlorotic and lacked vigor, and the disease picture was consequently complicated.

The most obvious effects arising from the differential light experiences appeared on those hosts that had a measure of genetic resistance. The symptom picture here, under medium and low light, tended toward one of susceptibility rather than resistance, and the small fleck-like lesions earlier regarded as typical of the response of the resistant host became large and diffuse. At the same time the fruiting rhythm was to some extent displaced and pycnidia matured several days later than on the diseased checks maintained in full light.

In view of these observations it is felt that the plasticity of the *Septoria* reaction as demonstrated on resistant hosts predisposed to low light intensities is such as to warrant the very discrete use of lesion size as a criterion for measuring the genetic resistance of host material.

Influence of Humidity

Plants were inoculated with spore suspension and incubated for varying lengths of time at saturation under lamp chimneys before transfer to humidities of lower values. The progress of the germination of the spores was observed at intervals from leaf samples of the material under test.

Experiment 1

Twenty-five tomato plants (Bonny Best) were atomized with spore suspension and placed under constant conditions of temperature at 25° C. in a saturated atmosphere maintained under glass chimneys. Pairs of plants

were removed from under the chimneys at 10- to 12-hr. intervals and exposed to controlled conditions in which the R.H. was 85%. Microscopic examination of cleared samples of leaves taken from such plants showed germination not to have taken place before the end of 40 hr., while penetration was beginning for the most part only in those leaves incubated for 48 to 50 hr.; a few penetrations were occasionally seen at the end of 40 hr. Typical leaf spotting with pycnidial production occurred only in those plants which had been in the incubation chambers at saturation for at least 48 hr. Increasing the time at saturation slightly beyond the 48-hr. period increased the number of lesions that eventually developed, while prolonging the period at saturation to 96 hr. brought about a type of "mass infection" with an apparent fusion of lesions and a rapid necrosis that destroyed the host before sporulation could occur.

Experiment 2

The procedure of Experiment 1 was repeated, the only change being the lowering of the relative humidity of the environment to which the plants were exposed subsequent to incubation, to approximately 58%. Again the period of approximately 48 hr. at saturation was required to ensure infection. The overall infection proved, however, to be considerably lighter at this reduced humidity.

Experiment 3

Having accumulated evidence which suggested that an interval of approximately 48 to 50 hr. was required for a spore to germinate and subsequently to negotiate the barriers offered by its host, it became of interest to test the behavior of the parasite on a series of inoculated plants which had been exposed to fluctuating conditions of humidity during this critical infection period. Could the spores, having once begun germination and the growth process, survive a period of unfavorable environmental conditions and yet retain sufficient vitality to enable them to establish themselves within the host when the conditions favorable for their activity were renewed?

Thirty-two plants, arranged in eight groups of four each, were inoculated and maintained at constant conditions of light and temperature in an environment in which the relative humidity was caused to fluctuate between saturation and 85% R.H. This fluctuation was brought about quite conveniently by maintaining saturation under lamp chimneys which covered the individual plants under observation, and removing the chimney after a given interval so as to expose the plant to the conditions of the constant-condition chamber in which the experiment was set up, the relative humidity being held there at 85%.

Immediately after atomizing them with a spore suspension the plants were placed under the alternative conditions of saturation or 85% R.H., and at irregular intervals thereafter, over a period extending through 60 to 70 hr., these conditions were alternated. Thus, an inoculated plant which for the first 12 hr. might have been maintained at saturation, would likely, during the

next period of 10 hr., experience no more than four hours at this high humidity, with this period being interrupted by several short experiences at the lower humidity. Similarly, if the initial experience of the plant should have been 12 hr. at 85% R.H., it would then be transferred for varying lengths of exposure to a saturated atmosphere. A different sequence of such fluctuations between the two humidities was given each set of plants, while the total time at saturation was varied among the sets to fall several hours each side of the "optimum" of 48 hr. (Fig. 1).

Reference to the summary in Table I will show the proportion of time of the sum total experience under fluctuating humidity that was actually spent under conditions of saturation. Among the sets under observation this period varied from 10 to 51 hr., but in no case was this of continuous duration, but rather was interrupted by periods of exposure to the reduced humidity of 85%, the duration of which ranged from 10 to 33 hr. Infection took place, as revealed by symptom expression on the 10th day of the experiment (approximately seven days after the plants had been removed to the constant conditions of 85% R.H.), only in those plants of Groups A, B, F, and H in which the total exposure to saturation had been not less than 40 hr.

It is also worthy of note here that the plants in Group F, whose total experience at saturation was 48 hr. but which had accumulated this experience under conditions which fluctuated between saturation and 85% R.H., were generally slightly more heavily infected than the controls which had been maintained for 48 hr. at 100% R.H. without fluctuation.

Experiment 4

Plants were inoculated with a spore suspension, placed immediately at 85% R.H. and left for 18 days; no symptoms developed under these conditions. However, all these plants became moderately to heavily diseased when they were removed to saturation for two days and subsequently observed over a period of eight days under conditions maintained at 85% R.H.

Similar results were obtained from plants previously exposed to a relative humidity of 55% for eight days, and then moved for a two-day period to a saturated environment. Such plants developed large spreading lesions which had very few pycnidia, and the plants were, on the whole, rather chlorotic after two weeks at 55% R.H. This spreading of lesions to give a target spot effect seems to be a symptom found rather generally at such lower humidities, although here it may, in part, be due to effects from incandescent lights as used in the growing chambers, since the tomato plant does not stand up well under such illumination.

A parallel experiment was carried out at an R.H. of 75%, with disease development occurring only after transfer to an incubation chamber which was held at saturation. Plants which had been given such an experience, when returned to 75% R.H. produced lesions which, while not of the discrete type

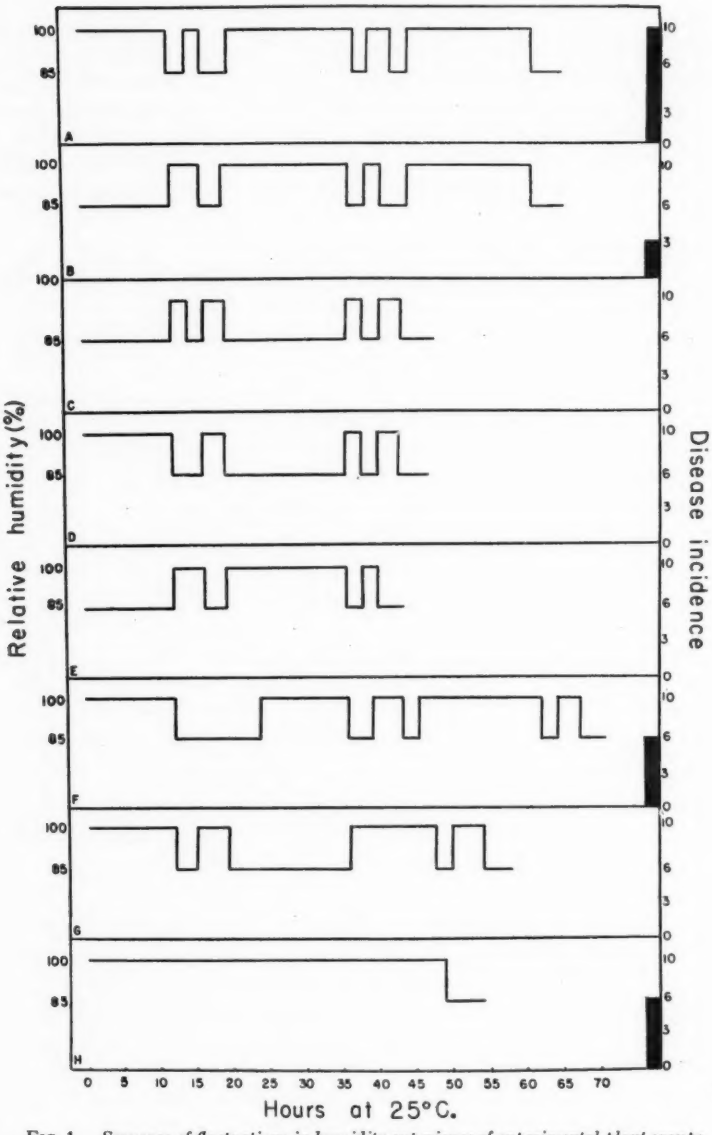

FIG. 1. *Sequence of fluctuations in humidity experience of experimental plant groups.*

found in plants infected at higher humidities, were much less of the spreading, nonfruiting character found in those plants which became diseased at 55% R.H. (Fig. 2).

TABLE I

INFLUENCE OF FLUCTUATING HUMIDITY UPON DISEASE INCIDENCE

Experimental groups of 10 plants each	Humidity experience and disease incidence			
	Total experience (hr.) at 85% R.H.	Total experience (hr.) at 100% R.H.	Sum total experience (hr.) under fluctuating humidity	Disease incidence
A	10	51	61	Heavy
B	20	40	60	Light
C	33	10	43	Nil
D	23	20	43	Nil
E	17	23	40	Nil
F	19	48	67	Medium
G	22	32	54	Nil
H	0	48	48	Medium

Thus, it would appear from the observations in these experiments that not only does *S. lycopersici* fail to develop and establish itself when denied an initial exposure to a saturated environment, but it is considerably influenced by a reduction in relative humidity even after the host has been successfully penetrated. This displacement from the humidity conditions optimum for parasitism is not necessarily occasioned by any visible adverse effect of the environment on the host, since at 75% R.H. where enlarged nonfruiting target spots appeared, tomato plants grow well and look thrifty.

Evidence here that the spores can survive conditions unfavorable for germination and penetration for at least eight days would indicate that short periods of dry weather are not necessarily limiting for *S. lycopersici* in the field. This would be especially true if during such periods of limited moisture, there were occasional nights in which the foliage was dampened by dew, since it is apparent that there can be a utilization of moisture by developing spores even though such periods may be separated by several hours of humidity conditions that would be considered as unfavorable for the onset of disease.

Experiment 5

While it was postulated above that a minimum period of approximately 48 hr. at saturation is required for infection, it has been rather convincingly demonstrated that these particular moisture conditions are not absolute, and that infection may take place at a relative humidity slightly below saturation. This was observed in a series in which the inoculated plants were placed immediately in 97 $\pm$ 1% R.H.; at the end of eight days symptoms of a heavy infection were being expressed.

INFLUENCE OF TEMPERATURE

Bonny Best tomato plants were inoculated with spore suspension and incubated at saturation at approximately 25° C. for 48 hr. The plants were

then distributed to constant-condition chambers whose temperatures were respectively 10°, 15°, and 20° C. A check group was left in the greenhouse where the temperature was above 20° C. at all times.

At the end of a 10 day period from inoculation, plants in the greenhouse and at 20° C. had developed symptoms which showed a normal amount of lesioning and good pycnidial development. Plants at 15° C. had an equal amount of infection but pycnidial development had been retarded and lagged considerably behind that at 20° C. and above, while the lesions tended to be large and spreading.

By the end of 16 days, plants at 15° C. showed large spreading lesions. Pycnidial development was fair, and, in contrast to the picture found at 20° and 25° C., the fruiting bodies were not confined to the central portion of the lesion area. No symptoms developed at 10° C.; the plants, though appearing healthy at this temperature, made little growth. Plants not developing any symptoms at 10° C., showed lesions and pycnidia within eight days after removal to 20° C.

Concurrent with the above observations, notes were taken on the effect of temperature changes at various intervals subsequent to the initial exposure at a particular temperature. It was found that while plants developed spreading lesions and were slow in producing fruiting bodies at 15° C., a transfer to the higher temperature of 20° C. before the fifth day produced a symptom picture not unlike that found in those plants maintained at 20° C. for the whole period. Plants moved to the higher temperature after a period longer than five days at the lower temperature did not show the same effect as noted above, but, rather, still produced a spreading type of lesion. We may recall here, for reference later, that the pycnidial primordia make their appearance on or before the fifth day, as was determined by our investigations into the histology of *Septoria lycopersici* infections; the effect of temperature in conditioning lesion size thus seems to be a function of its effect upon the rhythm of the fruiting cycle.

Perhaps the most interesting aspect of the influence of temperature upon disease expression is that noted when plants maintained at 20° C. were removed to lower temperatures of 15° and 10° C. In those instances where pycnidial formation had already well begun, there was no noticeable effect of the transfer to low temperatures; except for a slight retardation in the maturation of the fruiting bodies, the lesions develop normally. However, in those plants which were exposed to the lower temperatures on or before the formation of pycnidia, that is before the sixth day at the very latest, the effect was to promote lesion spread and restrict pycnidial production. This contrast in lesion character

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FIG. 2. *Large, spreading lesions on plant maintained under conditions of low humidity.*

FIG. 3. *A germinated spore effecting stomatal penetration with the development of an appressorium-like structure at the stomatal aperture.*

FIG. 4. *Infection of the differential host S25 with the virulent physiologic race 9F.*

FIG. 5. *Infection of the differential host S25 with the less virulent physiologic race 3A.*

PLATE I

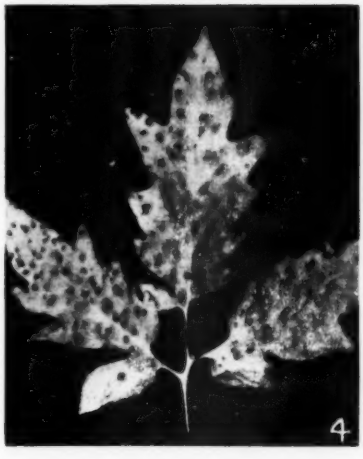

PLATE II

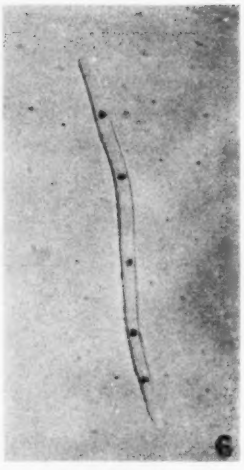

FIG. 6. *Spore of S. lycopersici treated with Feulgen to show nuclear condition (×1200).*

FIG. 7. *Nuclear division in rapidly growing mycelium as revealed after staining with Feulgen (×1200).*

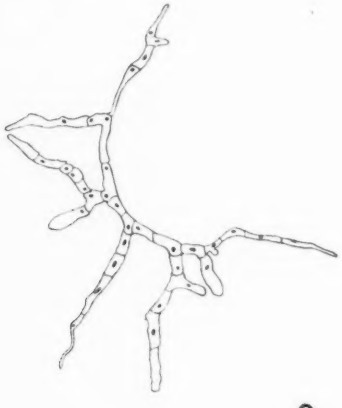

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FIG. 8. *A germinated spore stained with Feulgen showing but a single nucleus per cell (× 1200).*

as between plants transferred to the low temperatures after they have developed pycnidia and those transferred before this pycnidial stage has been reached is very striking. The explanation of this phenomenon probably lies in the fact that upon the formation of pycnidia and spores the contents of the mycelium making up the colony within any one infection locus flow to fruiting bodies and are there expended. Since low temperatures retarded the formation of pycnidia, the mycelium retains its vitality and so spreads into new tissue, thus enlarging the lesion.

Search for Perfect Stage

Several species of *Septoria* have been classified, on the basis of their perfect stage, as members of the genera *Leptosphaeria* and *Mycosphaerella*.

The writer observed perithecia of several genera on overwintered material, and all were picked off and cultured, but none of the ascospores so derived produced growth characteristic of *S. lycopersici*. In addition to this material from overwintered leaves, infected leaves kept in the refrigerator and periodically exposed to fluctuating temperatures, failed to produce the perfect stage of the tomato leaf spot fungus. Likewise agar cultures were stored at 5° C. for periods ranging from 12 to 25 weeks, and these were examined from time to time but a perfect stage was not found. However, on cherry extract agar, and on other media in which aerial mycelium was not too abundant, sclerotium-like growths were formed which gave the colony a distinct "dothidiaceous" appearance.

In addition to these observations, a careful examination was always made of any variant that appeared in culture. For the most part stock cultures were kept on tomato leaf extract agar, and on this medium the amount of vegetative development is at a minimum, while pycnidial formation seems unrestricted and spore production is heavy. Under such cultural conditions the fungus proved to be very stable with no changes in morphology at all apparent. However, on some occasions when cultures were grown on P.D.A., a white variant showing considerable aerial mycelium and no tendency to sporulate made its appearance. Such a variant could be subcultured by mycelial transfer, but even in pure culture it did not mature pycnidia, although a heavy stroma was formed. Such a spontaneous appearance of this white variant has since occurred in several of the original stock cultures when they have been grown on P.D.A., although there seems not to be an equal tendency among these cultures to produce this new form.

A white culture, comparable to the above variant, was received by the writer as *S. lycopersici*, from the "Bureau of Schimmelcultures" at Baarn, while the specimen obtained from the American Type Culture Collection similarly developed a white fungus in a portion of the slant. Attempts to infect plants with suspensions of mycelial fragments of such cultures have been unsuccessful.

Evidence for mutation in fungi, in the sense of genic alteration, is not readily adduced in the absence of a sexual stage. And yet, the white form of the fungus as described above came into view as a variant in a pure culture of

S. lyocpersici, and has made spontaneous appearance on several separate occasions. Further, it can be maintained in pure culture and shows no further indication of instability. It, then, is regarded as a mutant of *S. lycopersici*.

Concomitant with these observations, investigations into the nuclear condition of *S. lycopersici* were carried out with the aid of several techniques commonly used in cytological studies. That which proved to be most satisfactory was Coleman's modification (3) of the Feulgen method of nuclear fixation and staining. Earlier attempts to stain the nucleus of both germinated and nongerminated spores with such combinations of fixative stains as the acetocarmine and acetoorcein preparations gave results that could quite readily be misinterpreted should microscope equipment for extremely critical examination not be available. For example, with acetocarmine there were, in addition to the nucleus, several other bodies of equal and varying size that retained a stain that could not be distinguished from that of the true nucleus. Only with such critical optical equipment as the fluorite oil immersion was one led to suspect that material other than nuclear was being stained. This served to emphasize the fact that in the absence of a stain that is specific for nuclear materials, the true picture of the nuclear condition, especially as it exists in fungal preparations where the nuclei are generally extremely small, cannot be determined with any assurance of accuracy either as to the numbers of nuclei or their structural details.

In appreciation of the fact that the Feulgen technique was the only one that could be expected to give the true detail of the nuclear condition, it was used without further modification from that employed by modern cytologists in preparations of animal and plant material. The modification of Carnoy's fixative as suggested by Miss Hirsch (6) viz. one part glacial acetic acid, two parts absolute alcohol, and three parts chloroform, was an aid in clearing the spores and hyphae of oil globules. Hydrolysis was limited to seven minutes at 60° C., and both temperature and time were critical for the material at hand. Good preparations with deeply stained nuclei were obtained only with "activated" spores; that is, it was found necessary to stimulate the spores to a degree before they would respond to this staining technique. This activation was brought about by making up a spore suspension in nutrient and incubating it for 12 hr. or more; there was still no visible indication of germination at the end of this period and the only effect of this pretreatment was the enhanced stainability of the nuclei.

To prepare the material for Feulgen treatment the spores in suspension were smeared as a drop on a slide coated with albumen. After being dried, the slides were placed in the fixative for several hours. Although in most cases a good nuclear stain was obtained, the technique did not make for permanent preparations, and the color quickly faded. For that reason all photographs were made from freshly prepared mounts.

The multicellular spores may be said to have but one nucleus per cell (Fig. 6). This nucleus divides at the time of germination, often even prior

to the papilla stage of germ tube development; as the germ tube lengthens it becomes septate, and daughter nuclei become separated by the new cross walls (Figs. 7 and 8).

The exact origin of the nuclei in this multicellular spore is not known. One must recognize the possibility of such a spore being a "colony" of unrelated nuclei, as would be the case if these nuclei were not the product of mitotic division from a parent nucleus in the spore initial. However, single spore isolations made from any one strain of the fungus appear to be identical when compared on the basis of their pathogenicity, so that any variability as might exist among these nuclei of a spore is not readily apparent. The application of the Feulgen technique, or of any other accepted cytological techniques for that matter, has not been successful, as yet, in revealing the nuclear condition of the spore initials in the young, developing pycnidium. The apparent increased rate of metabolic activity in this region of fruiting promotes the retention of opaque cellular content within the mycelium and seriously disturbs the reaction of nuclear stains.

The nuclear condition of the vegetative mycelium is essentially the same as that indicated above. There is no suggestion of more than one nucleus in the normal, mature hyphal cell.

Physiologic Specialization

In order to demonstrate the existence of physiologic specialization in a fungus parasite one must locate hosts that display a differential reaction to the specialized races. In the present investigations, a range of hosts both within the genus *Lycopersicon*, and beyond it in related genera, were tested for their usefulness as differential material in determining whether or not specialization into physiologic races was existent in *S. lycopersici*. Hosts used in these tests included *Solanum tuberosum* L. (vars. Sebago, Green Mountain, Chippewa, and Katahdin), *S. carolinense* L., *Nicotiana tabacum* L., *Capsicum frutescens* L., *Physalis* spp., *Datura stramonium* L., *Lycopersicon esculentum* Mill. variety Bonny Best, together with various *L. esculentum* × *hirsutum*, *L. esculentum* × *peruvianum* crosses, as well as *L. hirsutum* Humb. and Bonpl., *L. pimpinellifolium* (Jusl.) Mill., and *L. peruvianum* (L.) Mill. themselves. These tomato stocks were produced as part of the breeding program carried out at the Horticultural Experiment Station at Vineland, Ont., while additional material was obtained from the U.S. Vegetable Breeding Laboratory in Charleston, S.C.

The search for physiologic specialization in *S. lycopersici* was initiated by making collections of leaf spot material from as many widely scattered locations in Canada and the United States as was feasible. This material was usually received in fresh condition and yielded vigorously sporulating cultures of the fungus. From each of such cultures one isolation was made by inoculating a slant of tomato extract agar with a spore suspension from an exuding pycnidium. This isolate then became an indexed stock culture and was used along with the others for comparative tests upon the series of differential hosts.

In addition to these cultures obtained from diseased material, three specimens of the fungus already in culture on artificial media, were obtained from three separate sources.

Inoculation tests were carried out by maintaining the plants, after they were atomized with spore suspension, under cloth cages kept moistened for at least 48 hr. Readings were taken at 24-hr. intervals. These frequent observations were felt to be necessary since it was important to note all such differences as might exist in length of incubation period and disease expression at early phases of lesion development. Final disease rating as to the overall severity of disease could usually be determined by the end of the 12th day, after which time secondary infections were being initiated.

In differentiating strain reactions in *S. lycopersici* it was believed that one or more of several criteria would be helpful; accordingly, in addition to length of incubation period, such characteristic features as size of lesion, amount of sporulation, and overall aggressiveness were noted. Each host series as it was tested for its inherent capacity for revealing the presence of strain differences was examined with such criteria in mind.

No differential reactions were noted on material that was not of the genus *Lycopersicon*. Uniform infection was obtained on all series except *Physalis* and tobacco, although sporulation was inhibited on potato under our cultural conditions. Large spreading lesions were general on *Datura*, while *S. carolinense* gave no indications of any strain differences among the cultures that were being used.

Similarly, no differences in pathogenicity as indicated by infections of Vineland stock were apparent, although derivatives of *L. hirsutum* × *esculentum*, as well as crosses of Red Currant (L. *pimpinellifolium*) × *esculentum*, showed a measure of resistance to *S. lycopersici*. When this material was turned up some special attention was given to it, and several replications of the inoculation tests were carried out, with comparable tests applied to field plots of the same stock. These latter tests were helpful in demonstrating the field resistance of material of both *L. hirsutum* and Red Currant stock, even after the seventh generation of selection for acceptable horticultural characters and resistance to *Cladosporium fulvum*.

It was only with the acquisition of some breeding stock from Dr. Andrus of the U.S. Vegetable Breeding Laboratory, that we began to find indications of strain differences amongst our cultures. According to Andrus and Reynard (1), the source of *Septoria* resistance used in their inheritance studies was a single plant selection derived from segregating lines of the Australian variety of tomato known as Targinnie Red. We were supplied with their basic stock which was a *Septoria*-resistant segregant of the above Targinnie Red selection known as M6, together with some derivatives (SWST and S25) that had shown promise as having resistance suitable for commercial stock.

Plants of M6, SWST, and S25 were inoculated and incubated at saturation in the usual way. Disease readings on the seventh day showed consistent

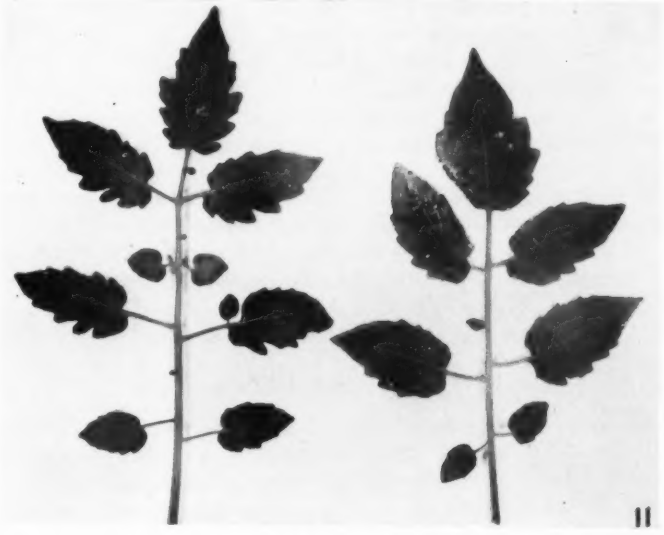

FIG. 9. *Heavy infection on tomato plant cultured under balanced conditions of nutrition.*

FIG. 10. *Light infection on tomato plant cultured under low nitrogen nutrition.*

FIG. 11. *Infection of the differential host M6 with the virulent race 9 F on the right, and with the less virulent race 3A on the left.*

infection on all plants under test, but differences in lesion size and overall aggressiveness were manifested throughout the lot. Close examination of these hosts showed that two extremes of disease reaction were being revealed, one consisting of a heavy mass infection with rather medium sized lesions, the other a light scattered infection with small, rather discrete lesions (Figs. 4 and 5).

At this point it is of interest to note that the quantitative differences between the two strains are most readily apparent on the susceptible Bonny Best, and it was in part the result of earlier observations of apparent differences in amounts of infection on Bonny Best arising from inoculation with these cultures (3A and 9F) that they were carefully checked upon the resistant hosts. On the other hand, differences in lesion size are not apparent on Bonny Best and are seen *only* on the resistant hosts. These differences are best expressed on the resistant hosts at about the 10th day; with the passage of time the smaller lesions of 3A seemed to enlarge, and, except for the fact that they are not so abundant as the 9F lesions, they have no specifically distinct features. Even in the extreme reaction of 9F there are not the large spreading lesions such as appear on the susceptible hosts; that is, the host still expresses a measure of resistance that tends to restrict lesion size (Fig. 11).

While some plants certainly have an intermediate amount of infection as compared to the heavy or light infections, and while there is a gradation of large lesions into smaller ones as resulting from infections of 9F and 3A, it has not been possible as yet to separate such reactions into group reactions. Rather, at the moment, we tentatively postulate, from the evidence as we have noted it on certain differential hosts, the existence of at least two physiologic races that differ from each other both quantitatively and qualitatively with respect to their pathogenic characters. Further research, with more satisfactory types of differential host material, will likely resolve the strain picture into more clear-cut differences; nevertheless, the task will be difficult in the absence of controlled environmental conditions during the infection period, and because of the further complication of modifying factors within the host which seemingly influence the expression of the resistant reaction.

Fungal Associations

During the course of these investigations, under the routine circumstance of isolating and culturing *S. lycopersici* from diseased field material, the presence of another fungus, a species of *Alternaria*, was noted. This fungus occurred with almost consistent regularity in the isolations from older lesions of leaf spot, and because of its rapid growth made the task of getting *Septoria* into culture somewhat more difficult. Apparently these two fungi were enjoying a coexistence in tissue that had been initially parasitized by one of them. Beyond this observation we offer no further information as to the nature of this fungal association, but in view of the few studies of such phenomena that have been made, we suggest that herein lies an opportunity for further work.

It is of interest also to note here, that in plate cultures of *S. lycopersici* contaminated with *Penicillium*, the stroma of the former may serve as a substrate upon which the latter grows in preference to the agar of the plate. Such a phenomenon is observed only in older cultures of *Septoria* which have become well established; if plates become contaminated at the time of inoculation with *Septoria*, the *Penicillium* will show abundant growth on the agar medium, and the *Septoria* will likely be crowded out.

Discussion

The problem of determining the limits of the parasitic potentialities of *S. lycopersici* resolves itself into a study of the plasticity of the interaction of host and parasite. The complicating factor here is the lack of a criterion which will measure host resistance and fungus aggressiveness, and at the same time make allowance for this plasticity. Lesion size has been the character most commonly used for measuring host response to parasitic action, since it is readily discernible and can be subjected to both qualitative and quantitative analysis. But with *S. lycopersici* it has been demonstrated that lesion size is to a large extent a function of the fruiting cycle of the fungus, and certain factors which condition the developmental rhythm of the fungus in turn affect the expression of host–parasite interaction. The greater part of these studies was centered about a consideration of these conditioning factors and the mechanism by which they operate.

The influence of the factors of temperature, humidity, and nutrition upon the expression of disease has been noted in our reports upon those experiments conducted under controlled conditions. The striking variability in lesion size in most cases could be related to the effect of one of these factors upon the growth and maturation of the parasite. Since the progress of colonization within any one lesion is a function of the fruiting cycle of the fungus, any condition, be it of environment, or of host or fungus physiology, which significantly displaces the rhythm of this cycle, in turn affects the character of the lesion through determining the extent of tissue colonization. Under conditions favorable for infection and subsequent parasitism of the host tissue, the fungus fruits in approximately 10 days. Histologic examination of the leaf spot at this time shows that the hyphal contents for the most part flow toward the fruiting body and are expended as spores; the developmental cycle of the parasite is complete, and no further colonization within this zone is possible. Under such conditions the size of the lesion can be said to be a function of host resistance and parasite aggressiveness, and as such have statistical significance when expressed as a disease rating. On the other hand, when conditions are such as to displace the rhythm of this fruiting cycle so that the process is not permitted to follow its normal course, hyphal colonization will continue and the lesions will enlarge. Lesion size under such circumstances is only relative to the conditions that prevail and so is not representative of the susceptibility of the host.

The effectiveness of *S. lycopersici* as a parasite of the tomato is readily apparent on the suceptible variety Bonny Best. Here the mycelium becomes intimately associated with the cells of the tissues colonized, though without any indication of the production of haustoria. Host response is one which Gaumann would describe as a normergic reaction with no sudden hypersensitive expression or histogenic changes; however, while the balance between the host and parasite is maintained long enough for sufficient maturation of the colony to allow for fruiting, this relationship between host and parasite is displaced at about the fourth day of infection. As Gaumann (4) would say, "the ratio between cause and effect has become disproportionate"; a necrosis of the host tissue is the result. Such interactions between compatible host and parasite show little evidence of the operation of any defense mechanism within the plant; rather it is worthy of note that lesions resulting from such a parasitic attack on compatible hosts very closely approximate in size those found in colony development when the fungus exists saprogenically on an agar plate.

To what extent the death of host cells is the result of their depletion by the parasite, or the action of a fungal toxin, or the effect of some product of host–parasite interaction is not known. Certainly there is no "action in advance", and the initial stages of infection are not marked by any drastic host response. Indeed, there is little indication of any pathogenic effect at all until about the fourth day of infection, at which time the pycnidial bodies begin to form. It is at this moment in the parasitic association of host and fungus that an acute reaction takes place. This is characterized by a brown discoloration in the regions of the pycnidia, followed by rapid collapse of the cells concerned. Evidently, the balance in host–parasite relationships, which was so strikingly manifest in the early stages of this association, is no longer maintained, and necrotic alterations in the infected cells rapidly cause death. This reaction, while most conspicuous in the areas of concentrated fungal activity, is by no means confined to these regions, but veinlets and adjoining mesophyll cells considerably removed from the centers of infection and beyond the locus of colonization become similarly affected, apparently by a pervasion of some toxic principle. It is to be noted also that no necrosis is found at those margins of the colonized tissues which are removed from the fruiting centers; apparently the "unbalance of the ratio between cause and effect" does not occur in these regions.

Hosts which are less compatible than Bonny Best react in most cases with a certain degree of sensitiveness that leads to necrosis and restricted colonization. In the penetration of *L. hirsutum* the first cells contacted are killed, and the fungus itself is apparently destroyed in the interaction since all attempts to isolate it from such lesions have failed. On the other hand, the infection of *L. hirsutum* var. *glabratum* (C. N. Mull.), though producing only nonfruiting lesions, allows for the recovery of the fungus from the necrotic spots. A somewhat similar situation is found in the infection of potato which, under our cultural conditions, also developed only nonfruiting lesions; from

these infected areas it was possible to isolate the fungus as late as 15 days after the initial appearance of the lesion. It would be interesting to know whether these two types of reaction, one as expressed on *hirsutum*, the other on the *glabratum* and potato, are in any way specific for the parasite concerned, or are merely general expressions of incompatibility. There is room for further research into the histopathology of such incompatibility reactions with a view toward an analysis of the actual defense mechanism operative within these hosts.

The genetics of the resistance to *Septoria* has been reported by Andrus and Reynard (1) to be of the monofactorial type which is inherited as a dominant character. Their work is supported by that of Locke (9), and all agree that the best sources of such resistance are stocks of *L. hirsutum* and *L. peruvianum*. This resistance in all cases is characterized by a restricted type of lesion much smaller than normal and bearing few or no pycnidia.

Our work was not directed toward a genetical analysis of the mechanism of the inheritance of such resistance, but rather involved the using of lines derived from both *hirsutum* and *peruvianum* stock which had been developed at the Vineland Horticultural Experiment Station for the express purpose of obtaining tomato varieties resistant to *Cladosporium fulvum*. Many such lines were tested by inoculating them with *Septoria*, but none showed any real promise either as truly resistant material or as differential hosts for the cultural strains of *Septoria* which we used.

In view of the dominant character of *Septoria* resistance as reported in the literature, it was not apparent why the Vineland crosses did not appear more resistant. Our *hirsutum* strain (P.I. 3833), as noted elsewhere, was very resistant, but the derivatives of it lacked this character; possibly the rigorous selection in one direction for *Cladosporium* resistance eliminated the lines which we sought, and yet, even in the F_1 of a Vineland *hirsutum* × Red Currant 11-22-15M cross, the plants developed a few sporulating lesions of the restricted type. It seems probable that there exists in *L. hirsutum* certain modifying factors which determine the degree of resistance that will be expressed in the hybrid derivatives. These observations may be placed beside those of Lincoln and Cummins (8) who note that "the size of the lesions and the number of pycnidia present in the F_1 is greater than in the parent strain".

Beyond those characters which might be said to be purely specific to the host, there are those inherent in the parasite which in turn condition host–parasite interaction. The variability of the pathogenic potential of *S. lycopersici* has been resolved into strain differences which, as denoted by their action on a series of differential hosts, are physiologically specialized into distinct races. Separation of such races has not yet progressed to the point where they can be identified from mixed infections upon any of the differential hosts used, though it is expected that once the measure of variability within any one race has been determined, it should be possible to make positive indentification of that race.

Yet, the presence of races that differ in their pathogenicity has been clearly demonstrated in inoculations with individual, pure cultures. Only those hosts with genetical resistance, derived from Andrus' original stock of Targinnie Red, have proved to be of use in differentiating those qualitative differences, although susceptible varieties of the tomato may exhibit differences in quantity of infection when attacked by the races under observation. On the basis of such qualitative and quantitative differences as exist when cultures of *S. lycopersici* induce disease on these differential hosts, it is concluded that there are at least two strains of the pathogen, one of which brings about enlarged lesions that show considerable sporulation on certain "resistant" hosts, or heavy disease and almost complete defoliation on susceptible hosts, while the other manifests a lack of virulence with restricted lesioning on resistant hosts, and but few lesions on the susceptible hosts. Such differences in pathogenicity are quite apparent, especially when the whole plants are brought together for comparison; it is believed that the tendency for the more resistant stocks to have a lower percentage of defoliation, even under heavy infection, partially obscures the quantitative differences noted in the susceptible varieties; in the latter the heavy defoliation following attack by the more virulent strain is very striking.

That there exists an intermediate strain, or strains, is also indicated in inoculations with cultures which give a disease incidence that is midway between the two strains discussed above. It is possible that these isolates as used are mixtures, since there seems to be considerable variability in lesion size as a result of infection from them. Further work in isolating from different types of lesions of such infections, together with the evolution of a wider range of differential host material should help in clarifying this problem of separating the several physiologic races that may occur within this species of *Septoria*.

Those aspects of parasitism which deal with prepenetration effects have been but touched upon in this study, and further investigations with a wider range of hosts is needed before a real evaluation of such effects can be made. In the same way the problem of fungal associations has merely been noted, with no attempt to enquire further into the phenomenon. It is felt that both of these problems have unlimited possibilities for careful research.

Further work with *Septoria lycopersici* as a representative of the pycnidiaceous group of the fungi is desirable. Here, apparently, is a group that is able to maintain its parasitic level of activity despite the fact that infection leads fairly rapidly to the necrosis of host cells. An important observation in this connection is the characteristically extreme localization of effect within colonized tissue; possibly it is this restricted necrosis that enables the fungus to complete its developmental cycle and reinitiate infections. Evidently there is maintained in the greater part of the colonized area, sufficient physiologic balance in the interaction of host and parasite to allow for the maturation of the pycnidia and the production of spores; yet there is little suggestion of an obligate relationship. At the same time it must be appreciated that the

saprophytic activity is at a minimum in the life cycle of *S. lycopersici*, and except for the possible absorption of some food materials during the final phase of pycnidial development, in which regions the host cells are dead, there is no evidence of saprophytism at all; however, the fungus is readily cultured on ordinary laboratory media. At the side of these considerations must be placed the additional suggestion of specialization toward a parasitic habit as is evidenced by the restricted host range of this fungus.

Such behavior makes it exceedingly difficult, if not impossible, to place *S. lycopersici* into any one category that might describe its physiologic activity. In the sense of de Bary, this fungus would fall either into the facultative saprophytes or the obligates, but in view of the plastic nature of *Septoria* whereby it exhibits both vigorous development on a saprophytic substrate and specialized parasitic activity on a living host, the limitations of such a classification are obvious. These studies but serve in this respect to emphasize the inadequacy of our classic methods of characterizing biological behavior.

Acknowledgments

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CARBONIC ANHYDRASE IN GREEN PLANTS[1]

By E. R. Waygood[2] and K. A. Clendenning[3]

Abstract

Carbonic anhydrase was found in leaf extracts prepared from 19 of 22 land and aquatic plant species examined. The most active preparations were obtained from *Spinacia oleracea* L., *Tetragonia expansa* Thunb., *Tropaeolum majus* L., and *Sambucus canadensis* L. Carbonic anhydrase is located in the leaf cytoplasm. Previously conflicting observations concerning its intracellular localization have been reconciled experimentally. Plant carbonic anhydrase is strongly inhibited by *M*/1000 azide, *M*/1000 cyanide, and *M*/2000 sulphanilamide and is weakly inhibited by 2,4-dichlorophenoxyacetic acid, diethyldithiocarbamate, and *o*-phenanthroline. The white zones of variegated *Tradescantia* leaves contain 50% less carbonic anhydrase than their green counterparts. Albino barley leaves contain 75% less carbonic anhydrase than normal barley leaves of the same size and age. The carbonic anhydrase content of green leaves kept in darkness for four and five days was lowered by 30–50%. Very young leaves contain less enzyme than mature leaves. These results are discussed in relation to the possible role of carbonic anhydrase in photosynthesis.

Introduction

Neish (13) provided the first positive evidence for the existence of carbonic anhydrase in green plants, using leaves of *Trifolium pratense* L., *Arctium minus* Bernh., and *Onoclea sensibilis* L. Roughton (14) and Burr (4) previously had failed to detect this enzyme in green plants, and later Mommaerts (12) was unable to demonstrate any carbonic anhydrase activity in *Heracleum sphondylium* L., *Lathyrus odoratus* L., *Spinacia oleracea* L., and *Elodea canadensis* Michx. Day and Franklin (6) tested 16 species including *Arctium minus*, but found anhydrase activity only in *Sambucus canadensis* L. Complete confirmation of Neish's work (13) was provided by Bradfield (2) who was able to demonstrate carbonic anhydrase in the leaves of a wide range of species and also in apparently inactive leaves after extraction with 0.01 *M* cysteine.

Earlier reports (2, 6, 13) on plant carbonic anhydrase indicate that the enzyme may be located in the chloroplast sediment (6), in the supernatant cell sap – cytoplasm (2) or in both the chloroplast and cytoplasm fractions (13). This disagreement has not been clarified by the recent work of Steeman Nielsen and Kristiansen (16) who found the enzyme to be restricted to the chloroplast sediment of cysteine leaf extracts from the CO_2-utilizing aquatic *Fontinalis dalecarlica* L., and the aquatic *Elodea canadensis* L. which can utilize either CO_2 molecules or HCO_3^- ions.

The present investigation was undertaken to settle the conflicting claims concerning distribution and intracellular localization of plant carbonic anhydrase and also to assess its potentiality as a photosynthetic enzyme. A brief report on some of our findings is being published elsewhere (17).

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Materials and Methods

Carbonic anhydrase activity was determined manometrically at 10° C. by measuring the rate of carbon dioxide evolution immediately after tipping 0.5 ml. of 0.2 *M* sodium bicarbonate – 0.02 *M* sodium hydroxide into 1.0 ml. of 0.2 *M* phosphate buffer (pH 6.8), in the presence and absence of enzyme solution (0.03 to 0.2 ml.). The reaction vessel was a 20 ml. rectangular Warburg vessel with two opposing sidearms, and without a center well. The vessel was fitted to the upper part of a Warburg manometer from which the manometer arm had been removed. A 12 in. length of pressure tubing connected the open end of this fixture to a stationary manometer supported outside the bath, and containing Brodie solution. Thus the reaction vessel could be shaken and tipped independently of the manometer, without removal from the bath. Air at atmospheric pressure was used as the gas phase. After five minutes' equilibration at a slow shaking rate, the vessel was tipped, and then shaken at 120 oscillations per minute. Constant volume measurements of the pressure increase were read on the open arm of the stationary manometer at 15-sec. intervals for 90 sec. One milliliter of 0.2 *M* phosphate buffer provided adequate buffering in the presence of all leaf extracts. The small total reaction volume of 1.5 ml. excluded diffusion effects in the presence of 1.0 units or less of carbonic anhydrase at the relatively slow shaking rate (120 r.p.m.) that was employed.

The ratio $\frac{R - R_0}{R_0}$ (11) was used as our enzyme unit (E.u.) where R = rate of catalyzed dehydration in the first minute at 10° C., and R_0 = rate of uncatalyzed dehydration. A single unit of enzyme is therefore the amount required to double the uncatalyzed rate of carbon dioxide evolution at 10° C. from 1.5 ml. phosphate–bicarbonate buffer mixture. The relationships of the catalyzed and uncatalyzed rates are represented graphically in Fig. 1, which shows the effect of adding 0.03 ml. of *Sambucus canadensis* leaf extract to the system. This effect was completely thermolabile. Repeated experiments have shown that boiled enzyme solutions have no effect, within experimental error, on the rate of carbon dioxide evolution (± 3% of the uncatalyzed rate). However, in those extracts having very low activity, the comparison was made with a boiled control, and in the experiments on cyanide inhibition, the uncatalyzed rate was determined in the presence of heated enzyme and cyanide. Our enzyme unit differs from that of Meldrum and Roughton (11) in that they used 2 ml. each of the phosphate and bicarbonate buffers at 15° C. The present unit includes the pressure changes from zero time, whereas Meldrum and Roughton omitted the first five seconds in their rate calculations. The apparatus employed in this study was designed to decrease the initial mixing error as much as possible, and a standard procedure was adopted for tipping, shaking, timing, and adjusting the manometer fluid.

Usually we prepared the enzyme solutions by grinding freshly gathered leaves in a mortar or mechanical juicer and expressing the juice through parachute nylon. All operations were performed either in a cold room or

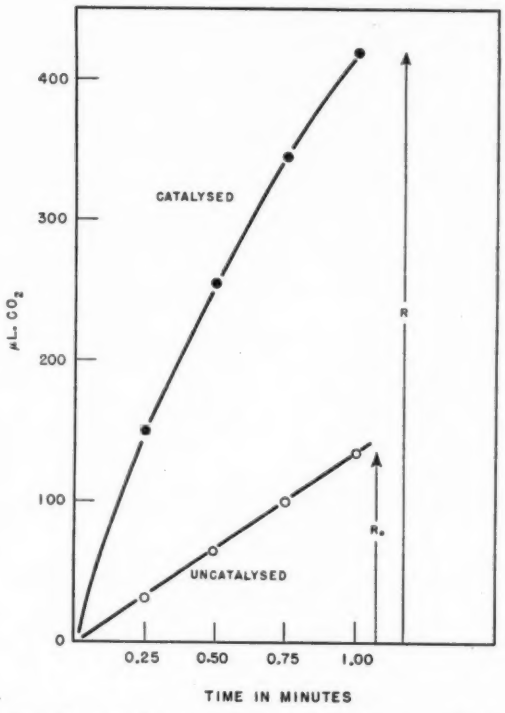

FIG. 1. *The effect of carbonic anhydrase from Sambucus canadensis on the dehydration of bicarbonate. Enzyme unit (E.u.)* $= \frac{R - R_0}{R_0}$.

with cold utensils. Each extract showing considerable activity was tested over a range of dilutions to determine the volume of extract required for activity measurements under enzyme-limited conditions (i.e. operating in the linear portion of the curve shown in Fig. 2).

For intracellular localization experiments the chloroplasts were separated from the cytoplasm by centrifugation at 15,000 g. for 15–30 min. Very finely dispersed chloroplast fragments were not deposited even after this centrifugation and in such cases chlorophyll was measured colorimetrically (5) on the resuspended residue and supernatant fractions to determine the distribution of chloroplast substance. After such vigorous centrifugation, the nuclei and mitochondria should be deposited with the chloroplasts, leaving only the cytoplasmic proteins and cell sap constituents in the supernatant solution. This was essentially true of all species studied with the exception of *Sambucus racemosa*, in which the cytoplasmic proteins were deposited with the chloroplasts.

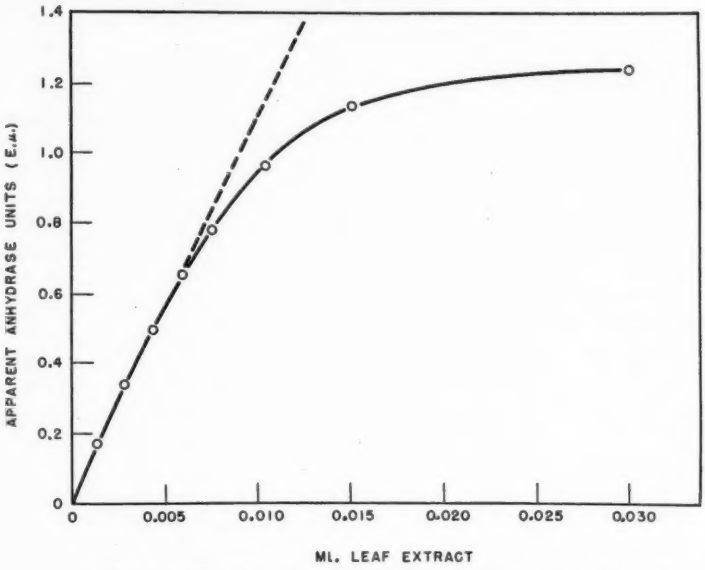

FIG. 2. *Effect of enzyme concentration on carbonic anhydrase activity.*

Results

Enzyme Concentration

The cytoplasmic fraction obtained from *Tetragonia expansa* leaves was tested for anhydrase activity at various dilutions, using 0.03 ml. enzyme solution in each test (Fig. 2). The data show that a linear relation, between enzyme concentration and activity represented as apparent enzyme units, is maintained only up to an E.u. value of unity. Above this value, the system is limited by diffusion. All determinations of enzyme activity reported hereafter therefore were calculated from activities observed under enzyme-limited conditions corresponding to the linear phase of Fig. 2.

pH Effects

It was not possible to change the pH of the reaction medium itself without involving a change in the method. Hence the pH relations of the enzyme were studied by incubating 1 ml. of *Tetragonia expansa* extract with 3 ml. of buffer solutions at various pH levels for 1–2.5 hr. prior to testing. The results (Table I and Fig. 3) show that the enzyme is about equally active after exposures to buffers of different pH within the range 5.0–7.0. Exposure to more acidic or more alkaline conditions resulted in lower activities when tested subsequently under standard conditions. The optimum pH range for stability of plant carbonic anhydrase is in the vicinity of the isoelectric point (pH 5.0–6.0) of the blood enzyme (7). The pH activity curve of the blood enzyme increases linearly from pH 6.2 to 8.1 or even higher (7).

TABLE I

INCUBATION CONDITIONS IN pH STABILITY EXPERIMENT

pH	Buffer components	Incubation time, hr.	Relative anhydrase activity, E.u.
3.8	0.1 *N* CH_3COOH	1.8	0.390
4.2	0.1 *N* CH_3COOH–CH_3COONa	1.5	0.710
5.0	0.2 *M* KH_2PO_4	1.0	1.030
5.7	H_2O	0.5	1.100
6.7	0.2 *M* KH_2PO_4–Na_2HPO_4	1.25	1.00
7.85	0.2 *M* Na_2HPO_4	2.25	0.805
8.4	0.02 *N* NaOH	2.5	0.775
8.7	0.2 *M* $NaHCO_3$–0.02 *N* NaOH	2.0	0.740
11.6	0.1 *N* NaOH	0.75	0.097

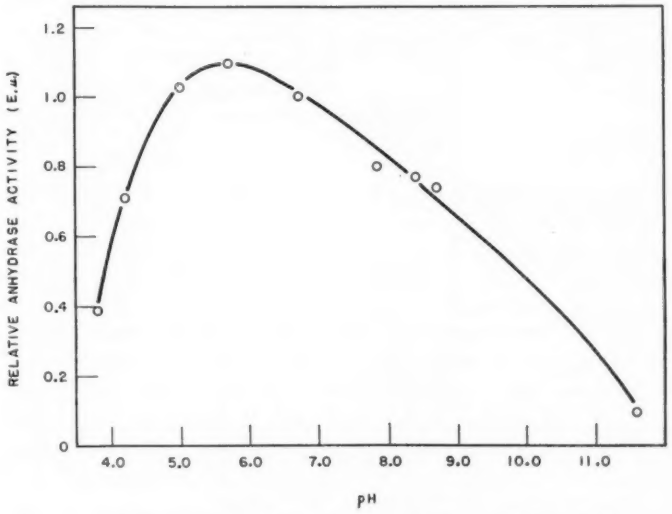

FIG. 3. *pH stability relations of plant carbonic anhydrase.*

The pH of the leaf extracts employed in the present investigation varied from 4.0 (*Kalanchoe coccinea, Rhus typhina*) to 6.2 (*Tradescantia, Sambucus canadensis*). The very low pH of some of the extracts probably contributed to their low anhydrase activity. However no simple relation was found to exist between carbonic anhydrase activity and pH of leaf extracts from different species.

Carbonic Anhydrase Activity of Different Species

Activity tests on extracts prepared from healthy mature leaves of 22 different plant species (Table II) shows that considerable intergeneric variation

TABLE II

CARBONIC ANHYDRASE ACTIVITY OF LEAF EXTRACTS FROM DIFFERENT PLANT SPECIES

Species	Common name	Volume of leaf extract employed, ml.	Dilution	E.u. per ml. original juice
Submerged aquatics				
Chlorella pyrenoidosa Chick	Chlorella Emerson strain	0.10	—	1
Elodea canadensis L.	Waterweed	0.20*	—	1.7
Myriophyllum spp.	Water milfoil	0.20*	—	0.5
Potamogeton spp.	Pondweed	0.20*	—	0.75
Cereals				
Hordeum vulgare L.	Barley	0.10	—	4
Panicum miliaceum L.	Millet	0.10	—	2
Triticum vulgare L.	Wheat	0.10	—	5
Other monocotyledons				
Tradescantia fluminensis Vell.	Wandering Jew	0.03	—	20
Chenopods				
Beta vulgaris L.	Sugar beet	0.03	4×	54
	Swiss chard	0.03	—	7.5
Spinacea oleracea L.	Spinach			
1. Stored 9 months at −40° C. (8)		0.03	4×	135
2. Lyophilized in *M* sucrose, stored 9 months at 4° C. (8)		0.05	—	20
Tetragonia expansa Thunb.	New Zealand spinach	0.03	4×	140
Other dicotyledons etc.				
Acer platanoides L.	Norway maple	0.10	—	1
Arctium minus L.	Burdock	0.10	—	9
Kalanchoe coccinea Welw.	Kalanchoe	0.10	—	1
Lycopersicon esculentum Mill.	Tomato	0.05	—	1
Nephrolepis exaltata Schott	Boston fern	0.10	—	0
Petroselinum hortense Hoffm.	Parsley	0.05	—	23
Rhus typhina L.	Sumach	0.10*	—	0
Sambucus canadensis L.	Common elder	0.03	4×	160
Sambucus racemosa L.	Red-berried elder	0.05	3×	90
Taraxacum officinale Weber	Dandelion	0.10	—	0
Tropaeolum majus L.	Nasturtium	0.03	4×	160

* *Extracted with cysteine.*

exists. The most active preparations were obtained from *Spinacia oleracea*, *Tetragonia expansa*, *Tropaeolum majus*, and *Sambucus canadensis*. Cysteine added to a final concentration of 0.01 *M*, as recommended by Bradfield (2) was required with some extracts, which otherwise showed no detectable activity. Stimulation of plant carbonic anhydrase activity with cysteine, which Bradfield reported to be a general phenomenon, was not observed on extracts from any of the "highly active" species, or from *Triticum vulgare*, *Hordeum vulgare*, *Rhus typhina*, and *Arctium minus*. The activity of the latter extracts

also was not increased when solid cysteine was employed, possible effects of dilution thus being avoided. However, we have observed 12% higher activity in cysteine vs. control *Tradescantia* extracts.

No common denominator such as a natural inhibitor was found to account for the different levels of carbonic anhydrase activity between species, as has been reported for the Hill reactions of isolated chloroplasts (5). Thus extracts of *Lycopersicon esculentum*, which were of very low activity, did not decrease the activity of *Petroselinum hortense* extracts when blended with them. Evidence of a natural inhibitor in *Rhus typhina* extracts was obtained by mixing them with extracts from *Sambucus canadensis*, the activity of the latter being reduced 30% below that of the controls. Since this effect did not persist when heated *Rhus* extracts were employed, it apparently was not caused by their acidity.

Extracts of *Spinacia oleracea* that had been stored for several months at −40° C. were among the most active preparations examined. Plant carbonic anhydrase thus does not deteriorate during lengthy storage at this low temperature. Lyophilized extracts, on the other hand, showed much lower activities, although still considerably higher than those of freshly prepared extracts obtained from several other species.

Intracellular Localization

The distribution of carbonic anhydrase activity between the chloroplast and cell sap – cytoplasm fractions, compared to the activity of the original leaf extract of *Tetragonia expansa*, is shown in Fig. 4. In this experiment 0.03 ml. of a four times diluted extract, supernatant, and residue resuspended in its original volume was added to the phosphate–bicarbonate system. A single centrifugation at 15,000 g. for 15 min. sedimented 98% of the chloroplast substance leaving 80% of the carbonic anhydrase in solution. Only a cytoplasmic enzyme could show this distribution.

The distribution of the enzyme in these fractions from other species is given in Table III together with data indicating the relative distribution of chlorophyll.

Only the woody perennial, *Sambucus racemosa*, and the aquatic plants have the anhydrase activity restricted to the crude chloroplast fraction. Day and Franklin (6) had previously reported that carbonic anhydrase is confined to the chloroplasts of *S. canadensis*. Contrary to their results, our tests on juvenile-type leaves of *S. canadensis* showed most of the activity in the cytoplasmic fraction, whereas tests on leaves collected from the previous season's wood showed the activity distributed equally between chloroplasts and cytoplasm. Apart from the above mentioned species, the carbonic anhydrase was recovered mainly in the cytoplasm.

A clean separation of the chloroplasts and cytoplasm cannot be expected by a single high speed centrifugation owing to the opportunity for adsorption on the chloroplasts. If adsorption is responsible for the low anhydrase activity

observed in most chloroplast sediments, then repeated washing and centrifuging should effect its removal at least partially. It was found that 33% of the carbonic anhydrase in *Tradescantia* chloroplasts (15 E.u. per ml.) could be

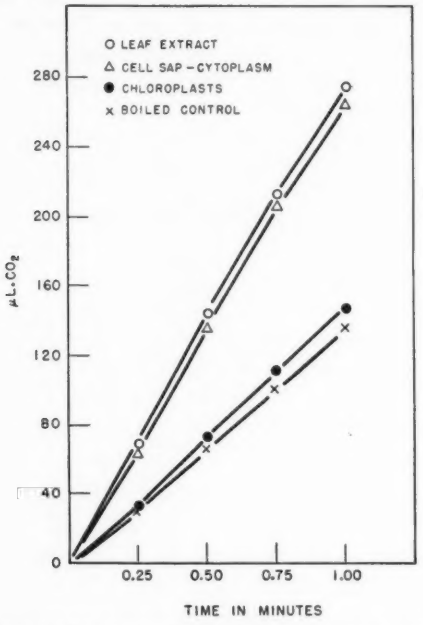

FIG. 4. *Activity of cytoplasm and chloroplasts of Tetragonia expansa compared to that of whole leaf extract.*

transferred to the supernatant by a single washing with water, and 53% by two washings. In neither case was there any contamination of the supernatant fraction with chlorophyllous material. The gain of activity by the supernatant fraction was accompanied by a corresponding loss of activity from the chloroplast sediment, the total number of enzyme units remaining unchanged. From experiments of this kind, it is concluded that the usually low carbonic anhydrase activity of chloroplasts separated by centrifugation is caused by adsorption.

The chloroplast fraction of *S. racemosa* was exceptional in that only 10% of its high anhydrase activity could be removed by one washing, and 17% by two washings (Table IV). The original supernatant fraction for this species contained only 2 E.u. per ml. Negligible protein was precipitated in this supernatant fraction on adding an excess of acetone or on boiling, as compared to the heavy protein precipitates obtained in the supernatant fractions of other species that contained the bulk of the carbonic anhydrase activity. It appeared likely that in *S. racemosa* extracts, a tannin-like substance caused

TABLE III

LOCALIZATION OF CARBONIC ANHYDRASE IN CENTRIFUGED LEAF EXTRACTS

Species	Carbonic anhydrase			Chlorophyll		
	Extract, E.u. per ml.	Cell sap – cytoplasm, %	Chloroplasts, %	Extract, μgm./ml.	Cell sap – cytoplasm, %	Chloroplasts, %
1. *Triticum vulgare*	2.0	100	2.0	—	—*	—
2. *Tradescantia fluminensis*	17.8	97	5.0	—	0**	—
3. *Tetragonia expansa*	137	82	20.5	600	4.35	98.5
4. *Tetragonia expansa*	118	91.5	12.8	600	6.8	112
5. Resuspended residue of 4	34.4	81.5	25.5	600	4.5	100
6. *Spinacia oleracea*	135	86	14	760	5.3	94.7
7. *Tropaeolum majus*	66	90	10	—	—*	—
8. *Sambucus canadensis*						
1. Juvenile-type leaves	158	75	28	400	37	63
2. Leaves from previous season's wood	47	50	50	—	—*	—
9. *Sambucus racemosa*	72	1.0	100	—	0**	—
10. *Elodea canadensis*	0.1	0	100	—	0**	—
11. *Potamogeton* spp.	0.75	0	100	—	0**	—

* *Slightly greenish.*

** *Chlorophyll visibly absent.*

TABLE IV

REDISTRIBUTION OF CARBONIC ANHYDRASE IN *Sambucus racemosa* BY TREATMENT WITH ALKALI

Treatment	Final pH	Carbonic anhydrase content		
		Extract, E.u. per ml.	Supernatant, %	Residue, %
1. None	6.0	150	2	98
2. Washed residue	6.0	150	10	90
3. Twice washed residue	6.0	150	7	93
4. Residue made alkaline	10.0	150	33	67
5. Water extracted	6.2	150	2	98
6. Phosphate extracted*	6.95	150	8	92
7. NaOH extracted	8.90	160	84	16

* *Chlorophyll visibly absent from supernatants of Nos. 1–5. Chlorophyll in No. 6 = 380 μgm. per ml. juice, supernatant contained 21%, and residue 79%.*

all of the cytoplasmic proteins, including carbonic anhydrase, to be precipitated with the chloroplasts. Vegetable tanning proceeds most rapidly at low pH (9). If tannins were responsible for the complete removal of proteins from the supernatant fraction, provision of a high pH should partially or wholly prevent cytoplasmic protein precipitation and hence result in a redistribution of the carbonic anhydrase.

Essentially that was what occurred. Extracts of *S. racemosa* normally have a pH of 6.0 – 6.2. When the pH of the resuspended chloroplasts was raised to pH 10.0, followed by centrifugation, one-third of the carbonic anhydrase

was transferred to the supernatant fraction (Table IV). Data for water-washed chloroplasts are included for comparison. Initial extraction of the leaves with disodium hydrogen phosphate at a final concentration of 0.06 *M*, pH 6.95, resulted in a partial redistribution, whereas crushing fresh leaves in dilute sodium hydroxide (final concentration 0.06 *N*, pH 8.9) resulted in 80% of the carbonic anhydrase remaining in the supernatant (Table IV). This latter treatment resulted in the retention of sufficient protein in the supernatant fraction to yield heavy precipitates on the addition of an excess of acetone or boiling. The supernatant and residue could now be correctly referred to as the cytoplasm and chloroplast fractions respectively. When applied to the chloroplast sediments prepared from aquatic plants, this procedure abolished the very low activity of the chloroplasts, but a corresponding gain in the carbonic anhydrase activity of the supernatant could not be detected.

Albino Leaves

Bradfield (2) has previously reported that plants showing high carbonic anhydrase activity in their leaves do not show detectable activity in their roots. The results of a comparison of the carbonic anhydrase activities of extracts from albino vs. normal leaves of *Hordeum vulgare*, and of the white and green parts of variegated *Tradescantia* leaves are shown in Table V. Lower activity was observed in albino barley leaves as compared to normal green leaves of the same size and age. When extracted with 2 ml. 0.1 *M* cysteine per 18 ml. extract, the activity in the extracts from the green leaves was reduced, whereas that in the albino extracts could no longer be detected.

In two experiments with variegated *Tradescantia* leaves, the carbonic anhydrase activity of the white parts was 50–60% lower than the green tissue (Table V).

TABLE V

CARBONIC ANHYDRASE IN GREEN AND ALBINO LEAF TISSUE

Species	Carbonic anhydrase, E.u. per ml.		Chlorophyll, μgm. per ml.	
	Green	Albino	Green	Albino
Tradescantia fluminensis	20	7.5	220	20
Tradescantia fluminensis	10	5	—	—
Hordeum vulgare	4.0	1.5	680	2
Hordeum vulgare (extracted with cysteine)	1.5	0	—	—

Effects of Dark Pretreatment

Tropaeolum majus and *Petroselinum hortense* plants were held in darkness for four and five days respectively, after which leaves were detached and extracted for activity measurements. Some of the *Tropaeolum* leaves were rendered visibly chlorotic, whereas others were nearly as green as the controls.

Accordingly, two samples, one of green and another of yellowing leaves, were tested. The *Petroselinum* leaves showed no visible chlorosis. The results, including the chlorophyll contents of all samples, are shown in Table VI.

TABLE VI

EFFECT OF EXPOSURE TO DARKNESS ON CARBONIC ANHYDRASE CONTENT OF LEAVES

Species and treatment	Carbonic anhydrase, E.u. per ml.	Chlorophyll, μgm. per ml.
Tropaeolum majus		
In light	173	1680
In dark, 4 days (green)	100	1200
In dark, 4 days (yellowing)	72	320
Petroselinum hortense		
In light	205	410
In dark, 5 days (green)	142	380

The carbonic anhydrase content of both samples was depleted by the dark treatment of the leaves, accompanied by, but not necessarily correlated with, a corresponding chlorophyll depletion.

Age of Leaf

Very young leaves of *T. expansa* have been shown previously to be lower than mature leaves in Hill reaction activity (5). Similar samples of juvenile and mature leaves were tested for carbonic anhydrase activity. The extracts prepared from the young leaves were much lower in carbonic anhydrase activity, expressed either as E.u. per ml. of extract or per gm. fresh weight (Table VII).

TABLE VII

EFFECT OF LEAF AGE ON CARBONIC ANHYDRASE CONTENT OF *Tetragonia expansa*

Leaf age	Leaf length, in.	E.u. per ml.	E.u. per gm. fresh wt.
Young	0.5 – 0.75	118	53
Mature	2.0 – 2.5	144	103

These effects of leaf age, of dark pretreatment, and of albinism, as well as Bradfield's observation (2) seem to establish the association of carbonic anhydrase with active photosynthetic tissue. These observations also indicate the adaptive nature of plant carbonic anhydrase.

Effects of Inhibitors

The sensitivity of plant carbonic anhydrase to cyanide was determined before and after dialyzing *Tradescantia* supernatants against 0.01 *M* cysteine in the cold. The results are shown in Figs. 5 and 6 with additional experimental data given in Table VIII. The controls included cyanide at the same

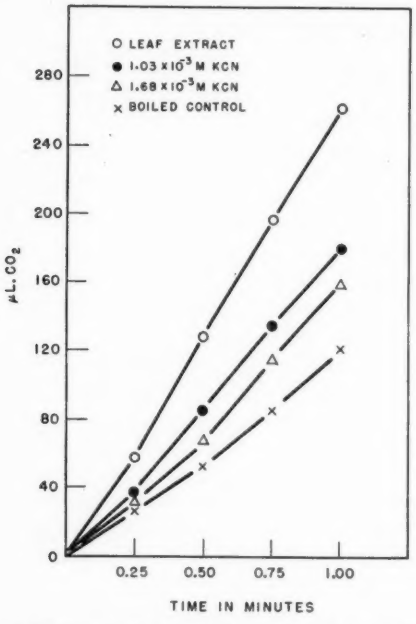

FIG. 5. *Inhibition of plant carbonic anhydrase by cyanide before dialysis.*

concentration as well as boiled enzyme, *M*/500 cyanide having been found to decrease the uncatalyzed rate of dehydration by about 10%. Cyanide (*M*/1000) inhibited the enzyme activity by 50% before, and 70% after dialysis. The inhibition by *M*/500 cyanide was correspondingly greater (Table VIII). The data in Table VIII show that some deterioration in the activity of the enzyme occurs over a 36 hr. period of dialysis. The level of activity in the control flasks was maintained by manipulating the dilution and amount of enzyme solution involved in the reaction. The greater sensitivity to cyanide following dialysis is attributed to the removal of substances having a greater affinity for cyanide than carbonic anhydrase. If this plant enzyme is a zinc protein, as has been suggested from analogy with the animal enzyme, the zinc moiety would have less affinity for cyanide than the more abundant free and bound metallic ions such as copper and iron. The present work has revealed a much greater sensitivity to cyanide than has been reported previously. Day and Franklin (6) reported no inhibition by cyanide, although

they did not state the concentration employed and it is possible that they were not working with an enzyme-limited system. Bradfield (2) obtained *ca.* 75% inhibition by $M/100$ cyanide. However, the plant carbonic anhydrase systems studied thus far are still considerably less sensitive to cyanide

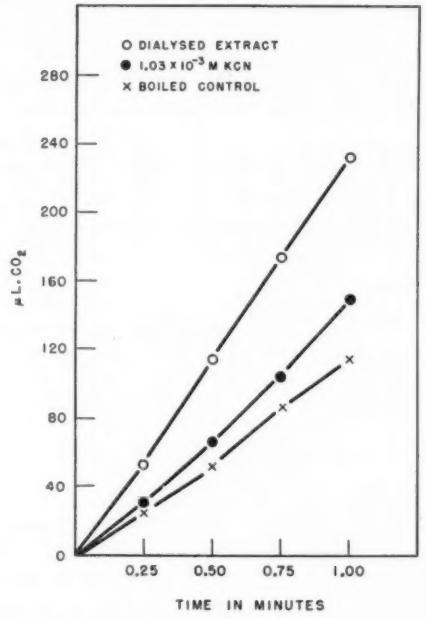

FIG. 6. *Inhibition of plant carbonic anhydrase by cyanide after dialysis.*

TABLE VIII

CYANIDE SENSITIVITY OF CARBONIC ANHYDRASE FROM *Tradescantia fluminensis*

Treatment	Inhibitor concentration ($\times 10^{-3}$ M)	Enzyme volume, ml.	Dilution factor	E.u. per ml.	E.u. per system	Inhibition, %
Undialyzed	1.03	0.2	3×	14.1	0.94	57.5
Undialyzed	1.68	0.2	3×	14.1	0.94	66.0
Dialyzed 12 hr.	0.89	0.1	0	7.8	0.78	53.5
Dialyzed 12 hr.	1.78	0.1	0	7.8	0.78	71.5
Dialyzed 36 hr.	1.03	0.2	0	3.4	0.68	73.0

than the animal enzyme. Carbonic anhydrase from blood is inhibited 85% by 4×10^{-6} M cyanide and completely by a concentration of $M/800$ (7). The physiological importance of the cyanide sensitivity of plant carbonic anhydrase is discussed later.

The effects of other inhibitors on carbonic anhydrase from various plant sources are shown in Table IX. All systems tested were enzyme limited. Plant carbonic anhydrase is more strongly inhibited by $M/1000$ azide than by

TABLE IX

EFFECT OF INHIBITORS ON CARBONIC ANHYDRASE

Inhibitor	Concentration, molar	Inhibition, %	Enzyme source
Sodium azide	1.05×10^{-3}	84	*Sambucus canadensis*
Sodium azide	1.05×10^{-3}	74	*Tetragonia expansa*
Sulphanilamide	5.2×10^{-4}	59	*Tradescantia*
o-Phenanthroline	1.05×10^{-3}	25	*S. canadensis*
2,4-Dichlorophenoxyacetic acid	0.96×10^{-3}	33	*T. expansa*
Sodium diethyldithiocarbamate	4.9×10^{-4}	18	*Tradescantia*
Iodoacetic acid	1.05×10^{-3}	6	*T. expansa*

cyanide, in agreement with the results of Bradfield who found *ca.* 90% inhibition by the same concentration of azide. It is also inhibited to a smaller extent by sulphanilamide, 2,4-dichlorophenoxyacetic acid, diethyldithiocarbamate, and *o*-phenanthroline.

Discussion

The present demonstration of carbonic anhydrase in the leaves of many species, in addition to those examined by Neish (13) and Bradfield (2), provides further evidence for the widespread occurrence of this enzyme in green plants. The failure of several investigators to find this enzyme in leaves is attributed to the techniques employed and to the particular species chosen, which might show only traces of activity in comparison with blood, the favored reference material. Mommaerts (12), who reported negative results, used a colorimetric technique in which the controls often showed 50% variation in an overall reaction time of 90 sec. Under such circumstances it would be exceedingly difficult to detect the enzyme in plant extracts, which are always of much lower activity than blood. Day and Franklin (6) reported negative findings for 15 species which included *Arctium minus*, a species found weakly active in the present study as well as by Neish (13).

The use of cysteine as recommended by Bradfield (2) and employed successfully on aquatic plant extracts by Steemann Nielsen and Kristiansen (16) has little or no effect on extracts obtained from many genera. What stimulation occurred from its use in the present investigation was never sufficient to increase the activity to that of a highly active species such as *Tetragonia*. The wide intergeneric variations in plant carbonic anhydrase activity cannot be explained by the pH relations of the enzyme or by natural inhibitors.

The data reported here show conclusively that carbonic anhydrase is located in the cytoplasm of the leaf cells of land plants. The apparent exception of *Sambucus racemosa* has been shown to be an artifact resulting from the presence

of a natural flocculating agent. On centrifuging, the cytoplasmic protein including carbonic anhydrase is deposited together with the chloroplasts. The presumed combination of vegetable tannin and cytoplasmic protein can be reversed with dilute alkali, and in its presence the cytoplasmic proteins remain soluble. On this basis we can explain the results of Day and Franklin (6) who found the enzyme confined to the chloroplast fraction of *S. canadensis*, although our locally gathered samples of *S. canadensis* did not differ from other land plants. Invariably in herbaceous land plants a variable but minor part of the anhydrase activity resided in the chloroplast fraction, which could readily be washed off with water.

The very low anhydrase activity of the aquatic plants was almost entirely confined to their chloroplasts. Alkali treatment inhibited the small activity present, making it impossible to ascertain whether flocculation of the cytoplasmic proteins had occurred, as in *S. racemosa* extracts. It is the authors' opinion that conclusions as to the location of this enzyme within leaf cells should not be drawn from observations on aquatic species showing less than 1% of the activity found in many land plants.

Interest in plant carbonic anhydrase stems from its possible role in photosynthesis. On theoretical grounds, Baas Becking and Hanson (1) concluded that hydration of carbon dioxide was the rate-limiting Blackman reaction. Similarly Burr (4) calculated that if HCO_3^- ions were utilized in photosynthesis catalytic hydration of carbon dioxide would be a necessity. Bradfield (2) found no detectable activity in the roots of plants whose leaves showed high activity. Our observations on albino and etiolated leaves as well as on leaves that remain green during lengthy dark treatments have shown large decreases in carbonic anhydrase activity in such tissues. These results, indicating that high carbonic anhydrase activity is associated with green leaves that are actively photosynthesizing, support the hypothesis that carbonic anhydrase is a photosynthetic enzyme. Very young *Tetragonia* leaves also show a considerably lower anhydrase activity than mature leaves, an ontogenetic difference that is similar to the capacity for Hill reactions (5) and presumably the capacity for photosynthesis (3).

The mechanism of photosynthesis can be separated into two component parts: (1) the photochemical production of reducing power by the chloroplasts; and (2) the dark assimilation of carbon dioxide at the expense of this reducing power. It has not been definitely established whether the intermediate stages of carbon dioxide reduction occur in the cytoplasm, in the chloroplasts, or on the surface of the chloroplasts. If the initial stages of carbon dioxide fixation are cytoplasmic, carbonic anhydrase would have to be located in this fraction to feed the dark carboxylation reactions. The known plant carboxylases (pyruvic, oxalacetic, oxalsuccinic, α-ketoglutaric and glutamic decarboxylases) have all been found to be located in the cytoplasm (18). None of these known plant carboxylases, however, have been established as participating in photosynthesis. Had carbonic anhydrase been restricted to the chloroplasts, then all subsequent steps involved in carbon

dioxide assimilation would be expected to occur within or on the plastids. The fact that carbonic anhydrase is located in the cytoplasm leaves open the possibility that carboxylases and other undefined enzymes which are involved in the assimilation of carbon dioxide may also be cytoplasmic.

Carbonic anhydrase is the first cyanide-sensitive plant enzyme which has been shown to have any connection with photosynthesis. The cyanide-sensitive reaction(s) of photosynthesis are generally believed to be connected with the fixation of carbon dioxide rather than with the photochemical production of reducing power by the chloroplasts (the Hill reactions of isolated chloroplasts which do not utilize carbon dioxide are not inhibited (10), whereas the dark fixation of carbon dioxide by living cells can be abolished by cyanide (15)). Carbonic anhydrase may well contribute to the cyanide-sensitivity of photosynthesis at saturating light intensity. It is considered unlikely that carbonic anhydrase is the only cyanide-sensitive enzyme connected with photosynthesis, otherwise the cyanide sensitivity of photosynthesis would disappear at moderate light intensities rather than near the compensation point.

The leaf extracts which showed highest carbonic anhydrase activity in this investigation had only 1/10th of the activity of human blood. It is interesting to compare, in this connection, the volumes of carbon dioxide and oxygen which are transported, per ml. of blood per hour in man, with the volumes of these gases which are transported in photosynthesizing cells, as ml. per ml. cell sap per hour. A survey of the physiological literature revealed basal rates of 3–6 ml. oxygen absorbed/ml. blood/hr.; during moderate exercise the rate increases to 20–30 ml., and at exhaustion exceeds 40 ml. O_2/ml. blood/hr. Willstätter and Stoll (19) have provided a wealth of data on the maximum photosynthetic capacities of leaves. Their values for normal green leaves, from 20 different species, range from 5 to 11 ml. carbon dioxide absorbed (or oxygen evolved) per gm. fresh weight of leaves per hour, and from 8–16 ml./ml. leaf sap/hr. (leaf fresh weight—dry matter). Winokur (20) has reported maximum rates of photosynthetic oxygen production in various *Chlorella* species of 13.6–41.4 ml. O_2/ml. packed cells/hr., or *ca.* 16–49 ml. O_2/ml. cell sap/hr. The maximum rates of carbon dioxide transport which have been observed in the cell sap of leaves and algae thus exceed the basal rate in the human blood stream, and are similar to the rate of carbon dioxide transport by an equal amount of blood in men doing moderate exercise.

Under field conditions, the respiration rate of green leaves is usually less than 1/10th of the rate of photosynthesis in saturating light. The rate at which carbon dioxide is respired does not exceed the uncatalyzed rate of carbon dioxide dehydration (4). Hence in respiring leaf tissue, no need for carbonic anhydrase arises. During photosynthesis at high light intensities, the carbon dioxide traffic exceeds the uncatalyzed rates of carbon dioxide hydration (4). If carbonic anhydrase has a role in the physiology of the green leaf, it would therefore appear to be connected with the process of photosynthesis.

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A STUDY OF THE ANTIBIOTIC ACTIVITY OF ACTINOMYCETES FROM SOILS OF NORTHERN CANADA[1]

By G. B. Landerkin,[2] Jane R. G. Smith,[3] and A. G. Lochhead[4]

Abstract

Of 660 cultures of actinomycetes isolated on a nonselective basis from soils from five locations in Northern Canada, 404 strains, or 61.2%, showed antagonism against at least one of eight test organisms consisting of five bacteria and three plant pathogenic fungi. The degree of activity ranged from slight to strong, with the number of test organisms inhibited by any single culture varying from one to six. In all, 49 different antibiotic spectra were observed. Activity against one or more of the pathogenic fungi was shown by 138 cultures, 20.9% of the isolates. The percentage of active cultures was greater, and the degree of inhibition much more marked, against *Helminthosporium sativum* than against *Fusarium culmorum* or *Fusarium lini*. The percentage of active isolates varied with the location and with the depth of the soil. It is suggested that the high proportion of actinomycetes with antagonistic properties occurring in northern soils may be related to the lower degree of plant development in such regions.

Introduction

Actinomycetes comprise an important group of microorganisms normally occurring in the soil. Though they are known to participate in many important biochemical processes in the soil, the actinomycetes have received rather less consideration that has been given to bacteria and fungi. More recently, however, recognition of the capacity of soil actinomycetes for producing antibiotic susbtances has focused greater attention on this group of microorganisms. In addition to their ability to form antimicrobial substances of highly important therapeutic application, there is evidence suggesting that, by virtue of the antagonistic effects which they exhibit towards other organisms they may participate actively in establishing the microbiological equilibrium in soil and may even be a factor in affecting the incidence of certain soil-borne plant diseases. The antibiotic properties of the actinomycetes have been well presented by Waksman (16, 17) while the antagonistic relationships of this group and other groups of soil microorganisms to plant pathogenic forms have been summarized by the same author (16) and in other reviews (3, 4, 13, 19).

Surveys of antibiotic actinomycetes in soil have shown antagonistic forms to be widely distributed. Of 80 strains isolated from a variety of Russian soils, Nakhimovskaia (11) reported 47 or 59% to be active against a variety of Gram-positive bacteria. In a study of 244 cultures, Waksman *et al.* (18) found 43% to possess some antagonistic properties. Emerson *et al.* (1) found 52% of their isolates of actinomycetes to show some inhibitory activity against at least one of five test bacteria, and 46% active against at least one of six

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pathogenic fungi. Of 243 actinomycetes isolated from soil and compost material, 51% were found by Schatz and Hazen (14) to be antagonistic to one or more of four test fungi. Fogle and Allen (2) reported 36% of 70 actinomycetes to be antagonistic to *Rhizobium lupini*. Landerkin and Lochhead (7) found 24% of 393 actinomycetes to show activity against *Staphylococcus aureus*, while of another group of 188 actinomycetes 47% were active against the same test organism and 60% against two strains of *Streptomyces scabies*. The same authors (9) also reported an increased incidence in the rhizosphere of potatoes of actinomycetes antagonistic to *S. scabies*, though not of those antagonistic to *S. aureus* or *Escherichia coli*, following the incorporation of soybean plant material in scab infested soil, the beneficial effect on the subsequent crop being related to a change in the microbiological equilibrium resulting from the addition of the organic matter (12).

Though actinomycetes are known to occur in soils under widely varying climatic conditions, most of the investigations of antagonistic forms have dealt with soils of the temperate zones. However, the studies of Meredith (10) on Jamaican soils, of Johnstone (5) on soil from Bikini Atoll, and of Thaysen *et al.* (15) on soils of Trinidad indicate that antibiotic-forming actinomycetes may be widely distributed in tropical soils. Less information is at hand regarding soils from colder zones, and although actinomycetes have been noted in samples taken from northern regions where the subsoil is permanently frozen, no information appears to be available on the incidence of antagonistic forms from such areas.

In the summer of 1949 an opportunity was afforded to obtain samples of soil from several locations in Northern Canada, through the co-operation of the Division of Entomology, Science Service. The present report deals with a survey of actinomycetes isolated from five of these with reference to their antagonistic action against a variety of bacteria and plant pathogenic fungi.

Materials and Methods

Soil samples were taken at depths of 0–1, 5–6, and 10–11 in. at the following locations—Cornwallis Island (at Resolute Bay); bank of Yukon River, Y.T.; Dawson, Y.T.; Yellowknife, N.W.T.; and Rupert House, Que. The soil was placed in small sterile jars and forwarded by air to Ottawa for analysis. Five-gram portions were added to 45 ml. sterile tap water, and a series of further dilutions plated on Conn's asparagine agar and Waksman's nitrate sucrose agar. After five days' incubation at 26° C. isolations were made on a nonselective basis by picking all actinomycete colonies on suitable plates and making transfers to slants of Conn's agar. Not all samples yielded actinomycetes in large number; however, where possible 100 or more isolations were made from soils of each location, a total of 660 cultures being obtained. All isolates were purified by streaking on poured plates of Conn's medium and re-isolated for detailed study.

For a study of the antibiotic activity of the isolates eight test organisms were used (see Table I). These included five bacterial species chosen on the basis of their susceptibility to known antibiotics and thus serving in some measure as a means of comparison. In addition three pathogenic fungi were used representing types responsible for soil-borne crop diseases of serious economic importance, and for which possible application of antagonistic microorganisms deserves consideration.

The antagonistic effects of the growth of 660 actinomycetes upon the test organisms were determined by the technique described by Landerkin and Lochhead (6). The actinomycetes were spot inoculated, four to a plate, on Conn's agar and after five to six days' incubation at 26° C. each plate was flooded with 3 ml. of seeded agar medium appropriate to the test organism, as indicated in Table I. Methods of seeding varied with the test organisms.

TABLE I

TEST ORGANISMS USED IN MEASURING ANTAGONISTIC EFFECT OF 660 ACTINOMYCETES FROM NORTHERN SOILS

Cult. No.	Test organism	Seed agar medium	Incubation after seeding
490	*Staphylococcus aureus* (F.D.A. No. 209)	Yeast beef agar	37° C., 24 hr.
246	*Bacillus subtilis* (N.C.T.C. No. 2586)	" " "	" 24 hr.
117	*Escherichia coli*	" " "	" 24 hr.
499	*Pseudomonas aeruginosa* (A.T.C.C. No. 9027)	" " "	" 24 hr.
546	*Mycobacterium tuberculosis* (A.T.C.C. No.607, rapid growing)	" " " 3% glycerol	" 48 hr.
638	*Helminthosporium sativum* (P. M. Simmonds, No. Hel 5)	Potato dextrose agar	26° C., 4 days
659	*Fusarium culmorum* (P. M. Simmonds)	" " "	" 4 days
666	*Fusarium lini* (M. I. Timonin)	" " "	" 4 days

For *S. aureus* and *E. coli*, a 1% inoculum from a 24 hr. yeast beef broth culture was used, while with *B. subtilis* and *M. tuberculosis* broth suspensions prepared from five- to seven-day agar slants were employed with a rate of seeding of 1½% for the latter. In the case of the fungi, the five- to seven-day growth on a slant of potato dextrose agar was suspended in 10 ml. of 'Tween 20' (diluted 1–5000) and seeded at a level of 1½ to 2%. After further incubation suited to the seeded test organism, plates were examined for antagonism and the diameter of zones of inhibition measured.

Results

A summary of the antagonistic action of the 660 actinomycetes against the eight test organisms, based on the degree of inhibition, is presented in Table II (see also Plate I). The pattern of activity against the test bacteria is

PLATE I

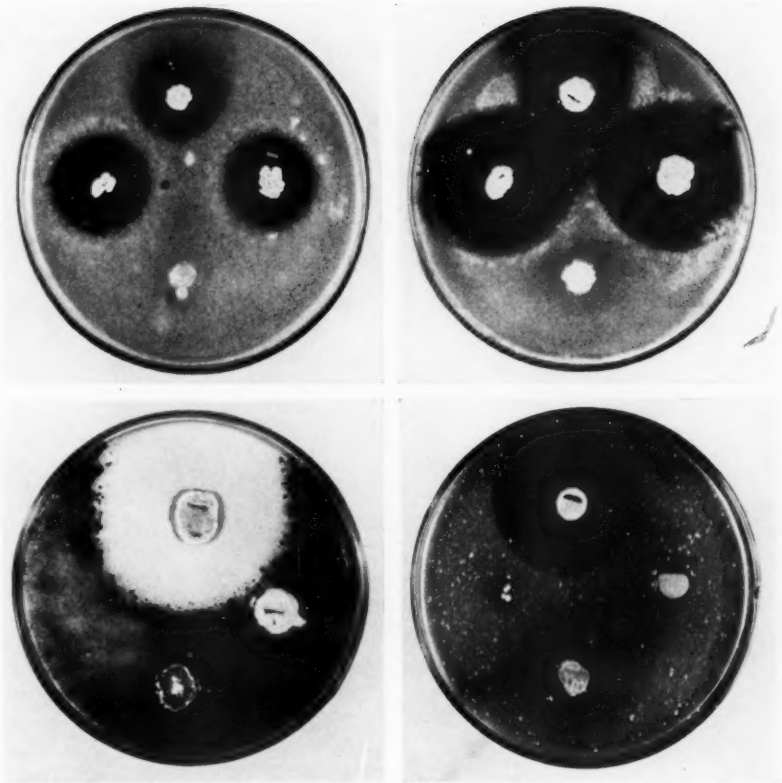

PLATE I. *Antibiotic effect of different actinomycetes against Fusarium lini (upper left), Fusarium culmorum (upper right), Helminthosporium sativum (lower left), and Mycobacterium tuberculosis (lower right).*

TABLE II

ANTIBIOTIC ACTIVITY OF 660 ACTINOMYCETES ISOLATED FROM NORTHERN CANADIAN SOILS

Degree of activity*	Number of cultures active against							
	S. aureus	*B. subtilis*	*E. coli*	*P. aeruginosa*	*M. tuberculosis*	*H. sativum*	*F. culmorum*	*F. lini*
3	140	140	5	0	8	35	3	2
2	84	96	13	0	28	43	26	14
1	87	90	36	3	93	39	53	46
0	349	334	606	657	531	543	578	598
No. active	311	326	54	3	129	117	82	62
%	47.1	49.4	8.2	0.5	19.5	17.7	12.4	9.4

* *3 = Zone diameter 30 mm. or more.*
2 = " " 20 mm.–29 mm.
1 = " " 10 mm.–19 mm.
0 = " " less than 10 mm.

essentially similar to that of actinomycetes from cultivated soils and conforms in general with the known susceptibilities of the organisms. As anticipated, greater antagonism was shown, not only in numbers but in degree, against the Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis*, than against the Gram-negative forms and *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* showing a characteristically high degree of resistance.

The scope of antibiotic activity is illustrated in Table III indicating the number of test organisms inhibited. Altogether 404 cultures, or 61.2% of the

TABLE III

ANTIBIOTIC ACTIVITY OF 660 ACTINOMYCETES IN RELATION TO NUMBERS OF TEST CULTURES INHIBITED

No. of test cultures inhibited	Active actinomycetes	
	No.	%
1	83	12.6
2	132	20.0
3	102	15.4
4	31	4.7
5	31	4.7
6	25	3.8
7	0	0.0
8	0	0.0
Total No. of active actinomycetes	404	61.2

actinomycetes, inhibited at least one of the test organisms, though none inhibited more than six of them. The percentage of cultures active against one or more test species from these Northern soils is therefore somewhat

greater than that so far recorded for isolates from cultivated soils, though accurate comparison is not possible on account of the different test organisms and methods used in various surveys.

The predominant antibiotic spectra shown by the isolates are presented in Table IV, which indicates the combinations of test organisms most frequently inhibited by the 660 actinomycetes. In all, 49 different spectra were observed, 19 of which were represented by a single actinomycete strain.

TABLE IV

PREDOMINANT ANTIBIOTIC SPECTRA OF ACTINOMYCETES FROM NORTHERN SOILS

Test organism	Antibiotic spectra in order of frequency											
S. aureus	+	+	−	+	+	+	+	−	+	+	−	+
B. subtilis	+	+	+	+	+	−	+	−	+	+	−	−
E. coli	−	−	−	−	+	−	−	−	−	−	−	+
P. aeruginosa	−	−	−	−	−	−	−	−	−	−	−	−
M. tuberculosis	−	+	−	+	−	−	−	−	+	−	+	−
H. sativum	−	−	−	+	−	−	+	+	+	+	−	−
F. culmorum	−	−	−	+	−	−	+	−	+	−	−	−
F. lini	−	−	−	+	−	−	+	−	−	−	−	−
Frequency	101	53	34	24	23	18	17	15	12	11	8	8

Of the 660 actinomycetes, 138 cultures or 20.9% were active against at least one of the three test fungi. As indicated in Table V the antagonistic

TABLE V

FUNGISTATIC ACTIVITY OF 660 ACTINOMYCETES FROM NORTHERN SOILS

Test organisms inhibited			Active actinomycetes	
H. sativum	*F. culmorum*	*F. lini*	No.	%
+	−	−	44	6.7
−	+	−	5	0.8
−	−	+	10	1.5
+	+	−	26	3.9
+	−	+	2	0.3
−	+	+	7	1.0
+	+	+	44	6.7
Total antifungal actinomycetes			138	20.9

types showed considerable diversity in antifungal effect, 59 being found active against but one fungus, 35 against two, and 44 against all three test species. From Table II it is seen that not only did a higher percentage of actinomycetes show antagonism to *Helminthosporium sativum* than to the two species of *Fusarium*, but also the degree of activity displayed by the active forms was

much greater against *H. sativum* than against *F. culmorum* or *F. lini*. The application of such antagonism against *H. sativum* as shown in Plate I is being further studied.

The distribution of active cultures isolated from soils from the various locations is shown in Table VI while the incidence of antagonistic types in

TABLE VI

ANTIBIOTIC ACTIVITY OF ACTINOMYCETES FROM SOILS OF DIFFERENT LOCATIONS

Location	No. of cultures	Percentage of cultures active against:							
		S. aureus	*B. subtilis*	*E. coli*	*P. aeruginosa*	*M. tuberculosis*	*H. sativum*	*F. culmorum*	*F. lini*
Cornwallis Island	71	15.5	32.4	1.4	0	2.8	9.8	1.4	1.4
Yukon River	173	63.0	63.0	1.1	0	42.7	32.3	27.7	21.4
Dawson	232	55.1	52.1	15.5	0	9.0	10.8	6.9	4.3
Yellowknife	105	24.7	33.3	6.6	2.8	11.4	15.2	9.5	7.6
Rupert House	75	48.0	49.3	10.6	0	26.6	17.3	9.3	8.0

samples taken at the three depths is given in Table VII. As regards location, the results suggest that the northern soils may differ considerably in the proportion of types antagonistic towards various microorganisms. Soil from

TABLE VII

ANTIBIOTIC ACTIVITY OF ACTINOMYCETES FROM SOILS AT DIFFERENT DEPTHS

Depth of soil	No. of cultures	Percentage of cultures active against							
		S. aureus	*B. subtilis*	*E. coli*	*P. aeruginosa*	*M. tuberculosis*	*H. sativum*	*F. culmorum*	*F. lini*
1 in.	253	36.8	39.9	2.8	0	18.2	17.0	10.7	8.3
6 in.	241	64.7	70.5	13.3	0	29.0	17.0	13.3	11.2
11 in.	166	37.3	39.2	9.0	1.8	7.8	19.9	13.8	8.4

Cornwallis Island yielded the lowest proportion of antagonistic forms while that sampled near the Yukon River showed in general the highest, and most noticeably against the plant pathogenic fungi. No explanation is offered for the differences noted, such as the higher proportion of actinomycetes antagonistic to *E. coli* in the soil from Dawson. It is of interest to note that in this case the site of the sampling was close to an inhabited area with consequently greater possibilities for addition of coliform organisms to the soil. However, as it is by no means established that organisms antagonistic to a specific type can be stimulated by the 'enrichment method', no definite conclusion is warranted. In Table VII there is some evidence indicating that at the 6 in. depth the soils harbor a rather higher proportion of antagonistic forms.

The relation of location and depth to antibiotic activity displayed by the actinomycetes is further shown in Figs. 1 and 2 in which an attempt has been

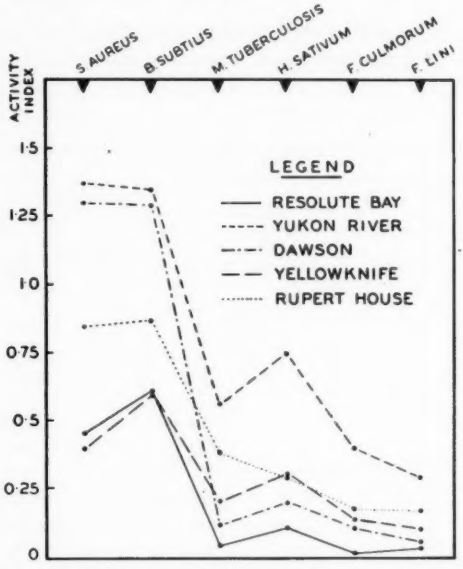

FIG. 1. *"Antibiotic index" of actinomycetes from soil of different locations.*

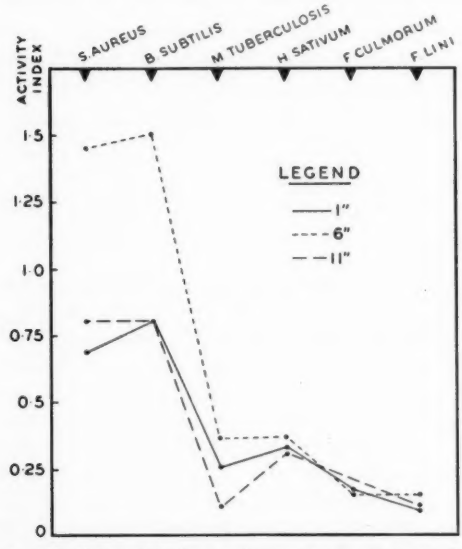

FIG. 2. *"Antibiotic index" of actinomycetes in relation to depth of soil.*

made to show the 'antibiotic index' of the soils with respect to the Gram positive bacteria and the three fungi, based on values obtained by 'weighting' the respective antagonisms according to the zones of inhibition displayed. Depending upon their degree of activity against the test organism in question, each isolate was given a value of 0, 1, 2, or 3 (cf. Table II). The sum of the products of these values and the frequency of their occurrence, divided by the total number of isolates, gave the 'antibiotic index', which represents thus the average inhibition exerted by the actinomycetes in any given sample or series of samples against a particular test organism. Though undue emphasis on such differences as shown in Figs. 1 and 2 is not warranted on the basis of a single year's survey, yet the results suggest strongly that soils may vary considerably in the mean antibiotic intensity of the microorganisms which they harbor and that this value may be related to the depth of the soil.

Discussion

From numerous studies of soils of different types and regions it is known that actinomycetes represent a numerically important group of microorganisms in both cultivated and virgin soils and may be found to be specially abundant under more alkaline conditions and in soils of high organic content. Their occurrence in soils from certain northern regions, therefore, is not unexpected. In earlier studies by one of us (8) on frozen soils of Southern Canada, it was found that the numbers of actinomycetes persisted without diminution throughout the period of frost, though, following the spring thawing of the soil, no pronounced increase similar to that observed for bacteria was noted. Though soil actinomycetes have a higher optimum temperature for growth than the bacteria in the same soil, they exhibit a high degree of resistance to low temperature which doubtless accounts for their occurrence in the soils of the present study which are exposed to extreme and prolonged cold.

It is apparent that northern soils may contain a high percentage of antagonistic forms, as high as, or higher than, found to occur in the soils from other regions. No reports have been noted regarding factors affecting the proportion of antagonistic organisms in soil, as distinct from total numbers. However, unpublished data from this laboratory indicate that the growing plant is a factor. Experiments with clover, flax, wheat, and potatoes have shown that in the rhizosphere of these plants, though the numbers of organisms are increased as compared with control soil, yet the percentage of bacteria and actinomycetes antagonistic to test organisms studied is decreased. It is therefore suggested that the relatively high percentage of antagonistic forms found in the soils from the northern regions may be related to the sparser vegetation and to the greater proportion of time during which no plant development occurs.

Acknowledgment

The authors wish to acknowledge with thanks the co-operation of Dr. T. N. Freeman and the staff of the Northern Insect Survey group of the Division of Entomology, Science Service, in securing the samples of soils used in this study.

Addendum

Since this manuscript was prepared for publication, Cooper and Chilton* have reported a survey of actinomycetes antagonistic to the root rot organism, *Pythium arrhenomanes*, from sugar-cane soils of Louisiana. The percentage of isolates antagonistic to the fungus ranged from 18.5 to 31.5 for the various soils examined. Comparison of the soils was also made on the basis of an "antibiotic index", strikingly similar to that described in this paper, as well as by an "antibiotic value" based on the numbers of actinomycetes per gram. The antifungal activity of the actinomycete population was found to vary with the soil reaction, showing a maximum in soils of pH 7.5.

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THE ROLE OF THE APHIDS, *BREVICORYNE BRASSICAE* AND *MYZUS PERSICAE*, IN THE SPREAD OF *XANTHOMONAS CAMPESTRIS*, THE CAUSAL ORGANISM OF BLACK ROT OF TURNIPS[1]

By Norma M. Stapleton[2] and L. A. McDermott[3]

Abstract

Turnip plants, heavily infested with the cabbage aphid, *Brevicoryne brassicae* (L.), and the green peach aphid, *Myzus persicae* (Sulz.), were inoculated with a virulent strain of *Xanthomonas campestris* (Pammel), the causal organism of black rot disease of turnips, and placed in special cages together with healthy turnip plants. Some aphids of both species were allowed to migrate naturally, and others were transferred mechanically from the diseased to the healthy plants, but no symptoms of black rot ever appeared in any of the latter plants. *X. campestris* was isolated from only 15% of the *B. brassicae* and from less than 1% of the *M. persicae* taken from the diseased plants. Aphids of both species were artificially contaminated externally with *X. campestris* by exposures to Petri plate and broth cultures of the pathogen. Internal contamination of other aphids of both species was accomplished by feeding them 0.2% aqueous dextrose solutions containing *X. campestris*. The pathogen was isolated from individuals in both groups of aphids thus contaminated, but none of the aphids in either group was able to infect caged, healthy turnip plants with black rot.

Introduction

Turnip growing is an important phase of farming in the province of Ontario. Within the last decade the average annual crop acreage in this province has been 40,000, with an average annual market value of around $4,400,000.

Recent heavy infestations of turnips with the cabbage aphid, *Brevicoryne brassicae* (L.), and the green peach aphid, *Myzus persicae* (Sulz.), together with some incidence of black rot in the turnip plants prompted this investigation to determine what role, if any, these insects played in the spread of this disease.

Black rot or bacterial wilt of turnips is a vascular disease caused by the bacterium, *Xanthomonas campestris* (Pammel). The bacteria gain entrance to the veins and vessels of the vascular bundles in the host plant where they multiply in the sap to such an extent as to occlude the passages. This causes a black discoloration of the veins and bundles of the infected plant and slow disintegration of the softer portions of the vessel walls. If a young turnip plant contracts the disease as a seedling or soon after being planted out in the field, the chances are that it will die soon or become a stunted, shrivelled, wilted specimen, producing no root.

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Contribution from the Department of Bacteriology, Ontario Agricultural College, Guelph, Ont. This paper constitutes part of a thesis submitted to the Graduate School of the University of Toronto in partial fulfilment of the requirements for the degree of Master of Science in Agriculture.

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Heavy infestations of aphids on young seedling turnip plants cause yellowing, wilting, and, in severe cases, death of the plants. On older plants the symptoms are not so severe. The cabbage aphid, *B. brassicae*, feeds on turnip plants in concentrated groups and produces localized yellow areas, while the green peach aphid, *M. persicae*, spreads over the plant and, in heavy infestations, causes a general yellowing of the leaves.

To date the only known methods of carrying *X. campestris* through the winter for primary inoculation in the spring is on the seed of the host plant and in diseased-plant refuse. Therefore, the importance of *B. brassicae* and *M. persicae* lies in their ability to effect secondary inoculations of turnip plants during the growing season.

Most of the literature on the importance of insects in the spread of black rot deals with this disease in cabbages and cauliflowers but not in turnips. What literature there is on the role of aphids in the spread of this disease is meager and controversial. Smith (11) suggested that aphids might carry the causal organism of black rot of cabbage. Brenner (1) isolated the black rot pathogen from an unnamed species of aphid which had been feeding on an inoculated cabbage plant, but he was unable to show that an aphid could successfully disseminate and inoculate the bacteria into the host plant. Clayton (2) reported experiments in which cauliflowers protected from insects remained healthy while others not so protected were heavily infected with black rot. This led him to conclude that insects were the major means in the dissemination of this disease with aphids the insects primarily concerned. Later experiments (3), however, failed to support this conclusion. He found that introducing worms, aphids, and thrips from diseased cauliflower plants either into cages of healthy plants or into cages containing diseased plants surrounded by healthy ones did not result in epidemic development of disease. Clayton believed that the cages prevented spread by wind-blown rain and by cultivation, ways in which he concluded that black rot was most frequently spread. Walker (12) reported that black rot of cabbage was spread by contaminated seed and disseminated by insects and snails which carried the pathogen from leaf to leaf. Leach, in his discussion of black rot (9), wrote: "Other workers have suggested various insects as possible vectors but have presented little or no experimental data. Until more conclusive evidence is available, it must be assumed that in nature insects are of little significance as vectors of black rot." Robbs (10) stated that *B. brassicae* is responsible for the greater part of infections of black rot, principally in cabbage, in Brazil. He claims that in the case of aphids the inoculation is direct and is made by the aphids feeding on infected plants and later flying or being carried by the wind to healthy plants.

Materials and Methods

Turnip Plants

Turnip plants of the variety Cannel's Purple King were used throughout this investigation. The seeds were planted in flats and allowed to germinate

PLATE I

FIG. 1. *Cages used to house turnip plants.*

in a greenhouse. The young seedlings were transplanted to pots and kept in the greenhouse until large enough for inoculation. Plants were inoculated when their roots were beginning to form. In the winter the plants were kept in cages in the laboratory, and the cages were placed outside in the open during the summer months.

Cages

The cages in which the turnips were kept were constructed of wood and celloglass and measured 24 in. by 18 in. by 18 in. (Fig. 1). The floor was wood and the sides celloglass with a 2½ in. wood base. On one side a wood inspection door fitted snugly into a wood frame. The tightly fitting but removable top was a framed piece of strong factory cotton.

Aphids

The culture of *B. brassicae* was obtained from turnip plants in the field and that of *M. persicae* from turnip plants in the greenhouse. The aphids were identified by Mrs. M. E. MacGillivray, Dominion Entomological Laboratory, Fredericton, N.B.

Plant Inoculations

Healthy turnip plants were inoculated with *X. campestris* to provide infected plants on which the aphids could feed. The culture of *X. campestris* was obtained from Dr. Armin C. Braun. It was one used by Elrod and Braun in their serological studies of the genus *Xanthomonas* (4, 5).

Plant inoculations were also used to identify bacteria isolated from plants and aphids and suspected of being *X. campestris*.

Spraying the leaves of healthy turnip plants with a suspension of the pathogen in a suitable wetting agent and root inoculations of healthy plants were both found to be unsatisfactory. Best results were obtained by midvein inoculations of the leaves. The midvein was stabbed at the base of a leaf with a hypodermic needle smeared with *X. campestris* from an agar culture. The tip of the needle was hooked slightly to help tear the plant tissue and thus ensure entrance into the vascular bundles of the plant. This method permitted the inoculation of all or just a few of the leaves of a plant.

Each leaf thus inoculated was cultured bacteriologically using Bacto Nutrient Agar (Difco). The isolates were tested serologically and by plant reinoculation. They were also examined microscopically. *X. campestris* was reisolated from over 75% of these inoculated leaves.

Identification of X. campestris

Colonies of suspected *X. campestris*, isolated from aphids and turnip plants, were identified by serological tests, plant inoculations, and microscopical examinations.

Suspicious colonies were tested serologically with *X. campestris*-immune rabbit serum using the slide or rapid agglutination test. Positive serological

tests were confirmed by plant inoculations, and in all cases the one test corroborated the other. Reisolations were made from the positive plant inoculations, and these in turn were reconfirmed by serological tests.

Hucker's modification of the Gram stain (7, 8) was employed in the microscopical examinations of the bacteria isolated.

Examination of Aphids for X. campestris

Gross examinations of aphids for the presence of *X. campestris* were made by crushing the insects in a few drops of sterile water in sterile Petri plates and then adding sterile Bacto Nutrient Agar (Difco) to the plates. The plates were incubated at 25° C. for 48 hr. and suspicious colonies tested serologically. All serologically positive bacteria were tested for pathogenicity by inoculation into healthy turnip plants.

Aphids were examined for the internal presence of *X. campestris*. They were first immersed for five minutes in a chlorine solution containing 150 p.p.m. available chlorine. This was found to be bactericidal for any of the black rot organisms present on the exteriors of the insects. The chlorine in the solution was then neutralized by the addition of 0.1 normal sodium thiosulphate; the aphids were cultured bacteriologically, and isolated colonies of bacteria were examined as described above.

Experiments and Results

Transmission of X. campestris by Aphids from Diseased to Healthy Turnip Plants

(*a*) *Natural Migration of Aphids from Diseased to Healthy Turnip Plants*

X. campestris was inoculated into the midveins of a few leaves on each of 24 turnip plants all heavily infested with both *B. brassicae* and *M. persicae*. Each inoculated plant was placed in a cage together with a healthy turnip plant. Care was taken to prevent the two plants touching one another. All the inoculated turnip plants wilted and died from black rot. Thirty days after the death of the inoculated plants the healthy plants still showed no symptoms of black rot although aphids did migrate to them from the diseased plants during the period.

(*b*) *Mechanical Transference of Aphids from Diseased to Healthy Turnip Plants*

Caged turnip plants, heavily infested with both species of aphids, were inoculated with *X. campestris*. When symptoms of black rot were evident, sections of the wilted leaves bearing aphids were cut out of each inoculated plant and placed on pieces of paper on the leaves of healthy, caged turnip plants. By transferring the aphids from diseased to healthy plants in this manner no damage was done to their mouth parts.

One hundred individuals of each of the two species of aphids were transferred from diseased to healthy plants in this manner and allowed to remain on the latter for 30 days. No symptoms of black rot appeared in any of the healthy plants during this period.

Examination of Aphids from Diseased Plants for X. campestris

(*a*) *Brevicoryne brassicae*

Turnip plants heavily infested with *B. brassicae* were inoculated with *X. campestris*. When symptoms of black rot were evident in these plants, aphids were removed from the wilted leaves and cultured bacteriologically. Twenty tests involving 10 aphids in each were thus carried out with this species of aphid.

Of the 200 *B. brassicae* thus examined, 30 or 15% were found to carry *X. campestris* externally and/or internally. These aphids were both the winged and wingless forms. When the winged forms were found to carry the pathogen, there were up to 10 colonies of *X. campestris* per Petri plate, whereas the count of those wingless forms carrying the pathogen was only one to three colonies per plate.

(*b*) *Myzus persicae*

Aphids of this species were examined in the same manner as *B. brassicae*. Forty tests with 10 aphids in each were carried out, and only three or less than 1% of these 400 wingless specimens of *M. persicae* were found to carry *X. campestris* externally and/or internally.

Nine to 10 days after inoculation of the turnip plants with *X. campestris*, the *M. persicae* showed a tendency to leave the plants or migrate. These migrants gathered around the edges inside the cages, often on the side towards the strongest light. Eleven hundred of these winged and wingless migrants were examined but none was found to carry *X. campestris*.

Transmission of X. campestris by Artificially Contaminated Aphids

(*a*) *Externally Contaminated Aphids*

Twenty aphids of both species were allowed to walk across an agar Petri plate culture of *X. campestris*, and then they were placed on leaves of a healthy, caged turnip plant.

About 400 aphids of both species were placed in a broth culture of *X. campestris*, removed almost immediately by filtration, and then distributed on the leaves of four healthy, caged turnip plants.

After a period of 45 days, no symptoms of black rot had appeared in any of the five healthy turnip plants infested with the aphids externally contaminated with *X. campestris* by these two methods.

(*b*) *Internally Contaminated Aphids*

Since *X. campestris* is not necessarily present throughout the entire vascular system of a diseased plant, an insect carrier need not necessarily harbor

the pathogen when on such a diseased plant. It is possible that when aphids from infected plants were transferred to healthy plants or were cultured bacteriologically they had not had the opportunity of picking up the pathogen. The only way to be certain that the aphids were carrying the pathogen internally would be to feed it to them artificially. This was done in a number of ways.

Aphids of both species were exposed to tap water and to various dextrose solutions in Petri plates and were found to exist for more than 48 hr. only in the presence of a 0.2% aqueous dextrose solution. This was taken to indicate that they fed on such a solution.

A group of both species of aphids was exposed to 5 ml. of a 0.2% aqueous dextrose solution containing *X. campestris*. After 48 hr., 40 ml. of a chlorine solution containing 150 p.p.m. available chlorine was added to kill the pathogen. The chlorine was then neutralized by the addition of 0.1 normal sodium thiosulphate. The aphids were rinsed in sterile water and dried on sterile filter paper. Some of these aphids were placed on the leaves of a healthy, caged turnip plant where they began to feed at once. Others were crushed and cultured bacteriologically, while the remainder were cultured without preliminary crushing. *X. campestris* was isolated from all the crushed aphids but not from any of those which had not been crushed. Thirty days later there were still no symptoms of black rot in the turnip plants on which the aphids, proved to contain *X. campestris*, had been feeding.

Another lot of both species of aphids was fed *X. campestris* in the same manner, but these insects were not treated with the chlorine solution. They were filtered directly from the dextrose solution and placed on the leaves of a young, healthy, caged turnip plant. All began to feed immediately, but 30 days later there was no infection evident in the plant.

The other methods of artificial feeding, i.e., in Petri plates containing absorbent paper saturated with the sugar solution containing the pathogen, through agar membranes, through wax membranes such as used by Fife (6), and through collodion membranes, all provided aphids carrying *X. campestris* internally but they were discarded for the one described above in detail because it proved to be the least cumbersome and the most efficient. However, none of the aphids fed the pathogen by any of these methods was capable of infecting healthy turnip plants with black rot.

Discussion and Conclusions

X. campestris can enter host plants through wounds made by insects, and one of the most important routes of infection is through the water pores at the edges of the leaves (11, 12). In moist, cloudy weather little dew-like droplets of water form at the water pores around the edges of the leaves. This is water of transpiration, and should *X. campestris* get into these droplets, it will find conditions suitable for entering the water pores of the plant from which it invades the veins to the vascular bundles. Insects are instrumental in introducing the pathogen into these water droplets.

Theoretically, aphids should be capable of spreading black rot. They wound plants by their feeding habits and could serve as carriers of the pathogen to the water droplets mentioned above. Under laboratory conditions, however, neither *B. brassicae* nor *M. persicae* was able to spread black rot from infected turnip plants to healthy plants. Only 15% of the former species and less than 1% of the latter species taken from infected plants carried *X. campestris* externally and/or internally. It was found possible to contaminate artificially both species of aphids externally and internally, but none of the contaminated aphids was capable of transmitting black rot to healthy turnip plants.

From the results of this investigation it would appear that these insects are not too important a means of spread of black rot in caged turnip plants. Although the conditions under which the experiments were carried out were as close to field conditions as cage experiments will permit, there is still the possibility that under field conditions these aphids might be a factor in the spread of this disease. The very fact that a few specimens of both *B. brassicae* and *M. persicae* did carry *X. campestris* labels these aphids as potential disseminators of black rot of turnips.

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NUTRITIONAL REQUIREMENTS OF *PSEUDOMONAS NIGRIFACIENS* AS RELATED TO GROWTH AND PIGMENT PRODUCTION[1]

By H. Katznelson and A. H. White

Abstract

The organic and inorganic requirements were determined for growth and pigment production by *Pseudomonas nigrifaciens*, an organism causing a reddish brown discoloration on the surface of mildly salted butter. Amino acids such as alanine, asparagine, proline, or glutamic acid were the best source of nitrogen for both growth and pigment production in a synthetic medium containing sodium chloride, inorganic salts, and glycerol; however, a carbohydrate was required only for pigment production in this amino acid medium. Na, K, Mg, S, P, and Cl or Br were also essential for both growth and pigmentation, larger amounts of these elements usually being required for synthesis of pigment than for growth. The dark brown-purple pigment produced was insoluble in all the common organic solvents hot or cold. With proline as a source of nitrogen a reddish-purple pigment developed which became deep purple then a deep brown purple. It is suggested that two pigments may be formed; one, a melanin-like dark brown substance originating possibly by polymerization of certain oxidation products of tyrosinase, the other, a dark purple condensation product of a secondary amine (such as proline) with the product (such as *o*-quinone) of a tyrosinase-catalyzed oxidation of phenolic substances produced by the organism.

Pseudomonas nigrifaciens is an organism described by White (18) that causes a black to reddish-brown discoloration on the surface of mildly salted butter. The defect is not apparent on the butter as it comes from cold storage in 56-lb. boxes, but usually appears after the butter has been cut into prints and held for five to nine days at temperatures commonly found in retail store refrigerators (4°–13° C.). Occasionally the discoloration occurs on butter in 56-lb. boxes which have been held a few days in storage at 3°–7° C. A similar defect has been reported by Hiscox (7) who isolated two strains of bacteria (*Pseudomonas* sp.) producing a bluish black pigment from the discolored areas of mildly salted butter (0.3–0.55% salt). Both investigators found that sodium chloride was required for growth and pigment production and that the latter was favored by low temperatures. The pigment was reported to be insoluble in the usual solvents and appears to be related to the melanins. The form of available nitrogen was of importance with respect to the nutrition of the organism and to its ability to produce the pigment (7).

Considerable work has been done on the influence of carbohydrates, sources of nitrogen, inorganic elements, reaction, and oxygen supply on pigment production by bacteria (1, 2, 3, 5, 6, 11, 13, 17) and no attempt will be made to review the literature. The object of this study was to determine the organic and inorganic requirements of *Pseudomonas nigrifaciens* in relation to growth and pigment production.

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Materials and Methods

Three strains of *P. nigrifaciens* were studied at first, after which the work was extended to include nine more, several of which were rough forms. The cultures were isolated from discolored butter samples, and were carried on nutrient agar containing 1.5% sodium chloride. Inoculum was prepared by suspending cells from a 72 hr. slant culture in 0.9% saline to give a turbidity equal to that of an arbitrarily selected standard consisting of cells suspended in formalin. One drop of inoculum was added to 10 ml. medium in 125 ml. Erlenmeyer flasks. Cultures were incubated for four and eight days at 7° C. in the early experiments; subsequently sets were kept at both 7° and 20° C. Growth and pigment production were estimated visually rather than photometrically owing to inability to separate the insoluble pigment from the cells in which it was partially retained. Throughout this work glassware was first treated with hot dichromate, allowed to stand over night in hot water, and then thoroughly rinsed in tap and distilled water. Baker's C.P. reagents only were used as required.

The organism was found to grow well and produce a dark brown-purple pigment often with an iridescent sheen in a basal medium containing inorganic salts (14), 1% glycerol, and 1.5% sodium chloride plus vitamin-free casein hydrolyzate. Although a carbohydrate was found to be unnecessary for growth, its presence enhanced pigment production in this medium. Glycerol was the most effective of the carbohydrates tested and glucose was superior to sucrose.

Nitrogen Requirements

When casein hydrolyzate was replaced with 19 amino acids excellent growth was obtained but less pigment production. The omission of each amino acid singly from the mixture did not affect growth or pigmentation significantly after eight days' incubation though slight differences were observed in the early stages. Media were then prepared with these amino acids and other organic and inorganic sources of nitrogen added singly at the rate of 6.7 gm. per liter. The results are presented in Table I and show striking differences depending on the nitrogen source. Inorganic nitrogen compounds and ammonium acetate and citrate were ineffective, ammonium tartrate supported some unpigmented growth and urea gave moderate growth without pigmentation. Of the amino acids used, glycine, serine, tryptophane, methionine, and threonine gave no growth; valine, leucine, aspartic acid, phenylalanine, tyrosine, cystine, lysine, histidine, and isoleucine gave slight to moderate growth with little or no pigmentation; however, alanine, asparagine, glutamic acid, arginine, and proline yielded excellent growth especially after four to eight days' incubation. This last group of amino acids with the exception of arginine and the possible exception of glutamic acid stimulated maximum pigment production after eight days' incubation. Proline was effective in the first few days of growth in stimulating pigmentation which was delayed with asparagine and alanine. Considerable strain variation with respect to both

TABLE I

INFLUENCE OF NITROGEN SOURCE ON GROWTH AND PIGMENT PRODUCTION AT ROOM TEMPERATURE BY *Pseudomonas nigrifaciens* (Strain P_3)

Nitrogen compound	Growth			Pigment production		
	2 days	4 days	8 days	2 days	4 days	8 days
Ammonium sulphate	±	±	±	0	0	0
" acetate	0	0	0	0	0	0
" citrate	0	0	0	0	0	0
" tartrate	±	+	++	0	0	0
Potassium nitrate	±	±	±	0	0	0
Urea	±	++	++±	0	0	0
Asparagine	++	+++	++++	+	+±	++±
Glycine	0	0	0	0	0	0
dl-Alanine	±	++	+++±	0	++	+++±
dl-Valine	+	+	+	0	0	0
l-Leucine	+	++	+++	0	0	0
l-Aspartic acid	+	+	++	0	0	±
l-Glutamic acid	+++	++++	++++	+	++	++
dl-Serine	0	0	0	0	0	0
dl-Phenylalanine	±	±	+	0	±	±
l-Tyrosine	±	+++	+++	±	±	+
l-Tryptophane	0	0	0	0	0	0
l-Cystine	+±	+±	+±	0	0	0
dl-Lysine	+	+	++	0	0	0
l-Histidine	+	+	+	0	0	±
l-Arginine	+±	+++	++++	±	±	±
l-Proline	++±	+++±	++++	+±	++±	+++±
dl-Isoleucine	±	+	++	0	0	0
dl-Methionine	±	±	±	0	0	0
dl-Threonine	0	0	±	0	0	0
Casein hydrolyzate	++++	++++	++++	+++	++++	++++

++++ = *Excellent growth or pigment production.*
+++ *Good* " " " "
++ *Moderate* " " " "
\+ *Fair* " " " "
± *Trace* " " " "
0 *No* " " " "

growth and especially pigment production was observed among the 12 strains. Several of these for example gave excellent pigmentation with glutamic acid as well as with alanine, asparagine, and proline and one produced relatively little pigment with proline alone.

As was to be expected the concentration of amino acid used affected both growth and pigment production (Table II). Little pigment was produced for example with concentrations of proline below 6.7 gm. per liter; but with asparagine and alanine moderate production of color was obtained even with 1.7 gm. per liter. With asparagine there appeared a tendency for lower pigment production with increasing concentration. The three compounds yielded excellent growth after eight days' incubation over the entire range of concentrations used though with alanine growth was somewhat slower during the first few days.

TABLE II

THE EFFECT OF CONCENTRATION OF NITROGEN SOURCE ON GROWTH AND PIGMENT PRODUCTION

Compound (gm./liter)		Growth			Pigment production		
		2 days	4 days	8 days	2 days	4 days	8 days
Proline	1.7	++±	++	+++	0	0	±
	3.3	++	++±	+++±	0	±	±
	5.1	++±	+++	++++	±	+	+
	6.7	++±	+++±	++++	++	++	+++
	8.4	++±	+++±	++++	++	++	+++
	10.0	++±	+++±	++++	++	++±	+++±
Asparagine	1.7	+	+++	++++	±	++	++±
	3.3	+±	+++±	++++	±	++	++±
	5.1	++	+++±	++++	±	±	++±
	6.7	++	+++±	++++	±	±	++±
	8.4	++±	+++±	++++	±	±	++
	10.0	++±	+++±	++++	0	±	++
Alanine	1.7	+	++	+++±	±	++	++
	3.3	+	++±	+++±	±	++	++±
	5.1	+	++±	++++	±	++	+++
	6.7	+	++±	++++	±	++	+++±
	8.4	+	++±	++++	±	++±	++++
	10.0	±	++	++++	±	++±	++++

++++ = *Maximum growth and pigment production.*
± = *Trace.*
0 = *None.*

More rapid growth and consistent development of pigment was obtained with all strains by combining asparagine, alanine, proline, and glutamic acid in different ways; this was especially true with proline and alanine which were therefore used, at the rate of 0.5% each, in all subsequent experiments.

Non-nitrogenous Nutrients

With a completely synthetic medium available, it was considered of interest to determine which of the non-nitrogenous constituents was essential for growth and pigment production. Accordingly media were prepared lacking glycerol, sodium chloride, salt solution A (dipotassium hydrogen phosphate, potassium dihydrogen phosphate) or salt solution B (ferrous sulphate, magnesium sulphate, manganese sulphate) with proline and alanine, or casein hydrolyzate, as the source of nitrogen. The data are summarized in Table III. In the presence of casein hydrolyzate maximum growth was obtained even in the absence of glycerol or sodium chloride but none in the absence of salts. However, pigment production was markedly reduced in absence of glycerol and even more so in the absence of sodium chloride; none was produced in the absence of both. The slight pigmentation in absence of added sodium chloride is due to the presence of sodium chloride in the neutralized casein hydrolyzate. However, of 12 strains tested, 10 gave no pigment in absence

TABLE III

ESSENTIALITY OF VARIOUS CONSTITUENTS OF THE MEDIUM FOR GROWTH AND PIGMENT PRODUCTION*

Medium	Growth	Pigment production
1. Basal** + casein hydrolyzate	++++	++++
2. 1 − glycerol	++++	++
3. 1 − NaCl	++++	±
4. 1 − glycerol + NaCl	++++	0
5. 1 − salt solutions A and B	0	0
6. Basal + proline + alanine	++++	+++±
7. 6 − glycerol	++++	0
8. 6 − NaCl	0	0
9. 6 − glycerol + NaCl	0	0
10. 6 − salt solutions A and B	0	0

* *After eight days at room temperature.*
** *Basal = salt solutions A and B, NaCl, glycerol.*

of sodium chloride; seven produced abundant pigment in absence of glycerol. When proline and alanine were used as the nitrogen source, it was found that glycerol was not needed for growth but was needed for pigment production. Sodium chloride and solutions A and B were required for both. Maximum growth and pigment production after eight days' incubation was obtained at a reaction between pH 7.0 and 8.5. There was no growth below pH 6.5.

Requirements for Inorganic Elements

When each of the elements represented in salt solutions A and B was eliminated singly from a medium containing glycerol, inorganic salts, proline, and alanine, it became evident (Table IV) that potassium, sodium, magnesium, chlorine, and sulphur were essential for both growth and pigment production.

TABLE IV

INFLUENCE OF INORGANIC ELEMENTS ON GROWTH AND PIGMENT PRODUCTION

Elements omitted	Growth			Pigment production		
	2 days	4 days	8 days	2 days	4 days	8 days
None	+++	++++	++++	+±	+++	++++
K	0	0	0	0	0	0
Na	0	0	0	0	0	0
Mg	0	0	0	0	0	0
Fe	++	+++	++++	0	±	++++
Mn	+++	+++±	++++	+±	+++	++++
P	+	+	+	0	0	0
Cl	0	0	0	0	0	0
S	0	0	0	0	0	0

PLATE I

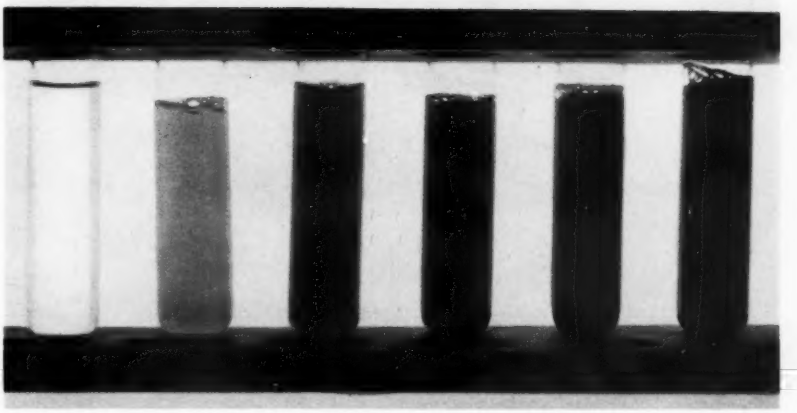

FIG. 1. *Influence of increasing level of potassium on pigment production by P. nigrifaciens. Colorless solution = 0.001% potassium chloride; increasingly black solutions = 0.005, 0.01, 0.05, 0.1, and 0.15% potassium chloride.*

Omission of phosphorus resulted in slight nonpigmented growth whereas omission of manganese was without effect on the organism. When iron was eliminated, growth was slightly depressed during the first four days and pigment production markedly delayed; both were at their maximum after eight days' incubation.

Experiments were then initiated to determine the effective concentrations of the various elements found to be essential.

Since sodium chloride was essential for both growth and pigment production it was added in amounts ranging from 0.1 to 5.0% to the synthetic medium. Excellent growth was obtained with concentrations from 1.0 to 3.0% and good growth even with 5.0% sodium chloride at eight days (Table V). However, 1.5% sodium chloride was the minimum required for

TABLE V

EFFECT OF SODIUM CHLORIDE CONCENTRATION ON GROWTH AND PIGMENT PRODUCTION

NaCl, %	Growth			Pigment production		
	2 days	4 days	8 days	2 days	4 days	8 days
0.1	0	0	0	0	0	0
0.5	±	+	++	0	0	0
1.0	+±	+++	++++	0	±	±
1.5	++±	+++	++++	+	++±	+++±
2.0	++	+++	++++	+	++±	++++
2.5	+±	+++	++++	±	++±	++++
3.0	+±	+++	++++	±	++±	++++
4.0	+	+++	+++	0	±	++±
5.0	0	±	+++	0	0	0

good pigment production which was noticeably reduced at 4%. Sodium chloride could not be replaced in the salts–glycerol–amino acid medium with lithium chloride, potassium chloride, magnesium chloride, potassium iodide, potassium sulphate, or sodium dihydrogen phosphate, but could be replaced with sodium bromide although this salt did not yield as dark a pigment as sodium chloride and, with some strains, no pigment at all.

Potassium and phosphorus requirements were determined in the sodium chloride – glycerol – amino acid medium by replacing salt solution A (dipotassium hydrogen phosphate and potassium dihydrogen phosphate) with disodium hydrogen phosphate and potassium chloride, magnesium by varying the amounts of magnesium sulphate and $SO_4^=$ by replacing magnesium sulphate with magnesium chloride and using varying amounts of sulphuric acid. The results are shown in Table VI and Fig. 1. Although excellent growth was obtained with 0.005% potassium chloride (as a source of potassium), larger amounts were required for pigment production at eight days. Similar results were obtained with disodium hydrogen phosphate (as a source of phosphorus). It may also be noted that considerably more magnesium was required to give optimum pigmentation than for growth but only slightly more $SO_4^=$.

TABLE VI

GROWTH AND PIGMENT PRODUCTION AS AFFECTED BY CONCENTRATION OF VARIOUS SALTS

K Cl, %	$MgSO_4$, %	Na_2HPO_4, %	$SO_4^=$, %	Growth		Pigment production	
				2 days	8 days	2 days	8 days
0.001	0.02	0.01	—	0	0	0	0
0.005	"	"	—	++	++++	±	++
0.01	"	"	—	++	++++	+	+++
0.05	"	"	—	++±	++++	+±	+++±
0.1	"	"	—	++±	++++	++	++++
0.15	"	"	—	++±	++++	++±	++++
0.1	"	0.001	—	+	+±	0	0
"	"	0.005	—	+±	+++±	+	+
"	"	0.01	—	+±	++++	+	+±
"	"	0.05	—	++	++++	++	++++
"	"	0.10	—	++	++++	++	++++
"	"	0.15	—	++±	++++	++	++++
K_2HPO_4			($FeSO_4$, $MnSO_4$ in basal medium)				
0.1	0.0002	—	"	±	++	0	0
"	0.001	—	"	±	++±	0	0
"	0.002	—	"	+	+++±	0	+
"	0.01	—	"	++	++++	0	+±
"	0.02	—	"	++±	++++	+	+++
"	0.03	—	"	+++	++++	+±	+++±
	$MgCl_2$		H_2SO_4				
"	0.02	—	0.0002	+	+	0	0
"	"	—	0.001	++	+++±	++	++
"	"	—	0.002	++±	++++	++	+++±
"	"	—	0.01	++±	++++	++	+++±
"	"	—	0.02	++±	++++	++	+++±
"	"	—	0.03	++±	++++	++	+++±

Some Characteristics of the Pigment

The pigment is insoluble in all the common organic solvents hot or cold. A culture solution dark brown purple in color, on filtration through a Seitz pad, yields a brown fluid, leaving a brownish purple residue which dissolves to some extent in warm concentrated hydrochloric or sulphuric acid but does not reprecipitate on dilution with water. It dissolves in concentrated sodium hydroxide to form a clear purplish-brown solution. This color and the brown color in the filtrate of the culture solution are not extractable with the common solvents. On heating, the purple tinge disappears and the solution becomes brown or light brown.

When proline is used as the organic nitrogen source alone or in combination with another amino acid such as alanine, a light reddish-purple color develops

with most cultures, becoming darker purple, deep purple, and finally dark brown purple. Tyrosine shows this effect to a slight degree. This reddish-purple pigment is extracellular and also insoluble in the usual solvents; it is thermolabile, turning to a light brown on heating or on standing after filtration through a Seitz pad.

Discussion

In common with other pseudomonads (2) *P. nigrifaciens* required no external supply of vitamins for growth. A carbohydrate such as glycerol was not required for growth in a synthetic medium containing amino acids indicating that the latter could serve as a source of carbon for the organism. Amino acids were the best source of nitrogen although moderate growth occurred with ammonium tartrate or urea and slight growth with some strains with ammonium sulphate. Hiscox (7) reported growth with ammonium citrate as the sole source of nitrogen, an observation which was not confirmed with the strains studied here. Not all the amino acids tested supported growth; glycine, serine, tryptophane, methionine, and threonine being completely inadequate.

For pigment production only certain amino acids were effective although Hiscox (7) obtained both growth and pigment production with ammonium citrate under certain conditions. Burton *et al.* (2) have also reported on the essentiality of specific amino acids for pigment production (pyocyanin) by *Pseudomonus aeruginosa*. However, for fluorescin production this organism can utilize ammonium succinate effectively (10). The inability of *P. nigrifaciens* to produce appreciable pigment in a synthetic medium containing 19 amino acids may be due to inhibition by one or more of these acids. Burton *et al.* (2) have shown for example that phenylalanine inhibits pyocyanin formation by *P. aeruginosa*.

Inorganic elements are of course required by all organisms for growth and multiplication; some, such as cobalt, copper, iron, manganese, and zinc are present in traces in most media, as in that used for *P. nigrifaciens*; others such as sodium, potassium, magnesium, phosphorus, sulphur, chlorine, or bromine are required in larger amounts by this organism since their omission from an otherwise adequate medium suppressed growth. However the omission of potassium or sulphate from a synthetic medium did not inhibit growth of *P. aeruginosa* (1).

The same ions required for growth of *P. nigrifaciens* were required for pigment production although in greater amounts. For example 10 times as much phosphorus (as disodium hydrogen phosphate) and magnesium (as magnesium sulphate) were required for good pigmentation as for growth. The essentiality of potassium, magnesium, sulphate, and phosphate for pyocyanin production by *P. aeruginosa* has also been reported (1), and of the last three elements for fluorescin production (9, 10) as well as for pigment production by *Serratia* (3). These three elements are frequently referred to as essential for pigmentation by bacteria (3, 4, 6, 11, 15). *P. nigrifaciens*

requires in addition, sodium and chlorine; bromine can replace chlorine completely for some strains, moderately for other strains, and not at all for still others with respect to pigment production.

Although the nature of the dark brown pigment has not been studied extensively it appears to be a melanin-like substance. The reddish purple color obtained to a very slight degree with tyrosine and much more abundantly with proline suggests a tyrosinase reaction to melanin via halochrome, a reddish substance which undergoes spontaneous oxidation to melanin (16). It is surprising, however, that so little pigment is produced in the presence of added tyrosine. Another course of pigment production catalyzed by tyrosinase has been reported by Jackson and Kendal (8) who showed that proline or hydroxyproline can condense with *o*-quinone to form a leuco pigment which is spontaneously oxidized to the colored form (purple to deep purple) by oxygen. The reaction involves an amino acid (with a secondary amine) catechol or homocatechol and the enzyme tyrosinase. It appears therefore that two pigments may be formed; one, a melanin-like dark brown substance, originating possibly by polymerization of certain oxidation products of tyrosinase (12), the other, a dark purple condensation product of a secondary amine (such as proline) with the product (such as *o*-quinone) of a tyrosinase-catalyzed oxidation of phenolic substances produced by the organism. The production of the deep purple pigment in absence of added proline may be due to the synthesis of this amino acid or closely related substances by the organism, followed by condensation. Further work is required to elucidate this problem.

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STUDIES OF CANADIAN THELEPHORACEAE

VII. SOME NEW SPECIES OF *CORTICIUM*, SECTION ATHELE[1]

By H. S. Jackson[2]

Abstract

A series of nine delicate species, for the most part inconspicuous forms, which seem related to the more typical members of Bourdot and Galzin's section Athele of the genus *Corticium* are described and illustrated. The general features of the group are discussed as well as the possible interrelations of the species described.

Introduction

Included in the present contribution is a series of nine delicate, thin, usually inconspicuous, occasionally subinvisible species, all presumably undescribed, which would for the most part have been included by Bourdot and Galzin (1) in their sections "Athele" of *Corticium* or *Gloeocystidium* or in group C) of their section "Ceracea" of *Corticium*.

Bourdot and Galzin define their section Athele as follows: "Ceraceous or subpellicular, dry adherent, trama formed of few hyphae, short or more often indistinct; spores generally clinging together in groups of 2–4, species living especially on dry wood or herbaceous debris, rarely on very decayed wood. The hyphae are fairly distinct in the first five species, generally indistinct in the others".

The first five species listed are not known to the writer except by description and the relation of some of them would seem doubtful; but if *Corticium aurora* Berk., *C. juncicola* Bourdot, *C. filicinum* Bourdot, and *C. Lloydii* B. & G. are taken as the more typical members of the section, they apparently form a quite natural group. To these should be added, in all probability, some of the ceraceous species included in group C) of their section "Ceracea". Others which seem related are scattered in other sections including *C. tulasnelloideum* Höhn. & Litsch. and perhaps some other of the rough-spored species included in their section "Humicola", and in *Peniophora*, a group of four species centering around *P. rimicola* (Karst.) Höhn. & Litsch. as recently discussed by Jackson (3). Such species of *Peniophora* as *P. clematidis* B. & G., *P. abietis* B. & G., and *P. lauta* Jackson should be taken into consideration as possibly related.

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Previous numbers in this series were published in Can. J. Research, C, 26 : 128-139; 143-157. 1948: in C, 27 : 147-156; 241-252. 1949: and in C, 28 : 63-77; 525-534. 1950.

[2] *The writer is indebted to Miss Elizabeth Ruth Dearden for assistance with the manuscript and for the drawings of Fig. 9, to Miss Charlotte E. Dill, for the preparation of the drawings for Figs. 1–8, to Miss Margaret H. Thomson for the preparation of the latin diagnoses, and to Dr. D. P. Rogers for critically reading the manuscript.*

Thus limited we have a group of species with a series of general characters more or less in common. Most of them are relatively inconspicuous forms, thin and delicate, adherent, often with little development of subiculum or with the subiculum indistinct. In consistency the fructifications vary from dry pruinose or crustose granular to subceraceous or ceraceous. The ceraceous or waxy gelatinous forms dry to a thin sometimes barely perceptible vernicose film as in some species of *Sebacina*. In some the subiculum consists merely of a few horizontal hyphae from which the basidia may arise directly from hyphal cells or on short branches. In some of the ceraceous species the subiculum may be fairly well developed, as in *C. tulasnelloideum* and its relatives, *C. pseudotsugae* and others, and in the *Peniophora rimicola* group. In these there may be an initial basal layer of horizontal hyphae but the bulk of the subiculum is obscure and appears to consist for the most part of dead gelatinized hyphae with a surface layer of living hyphae from which the basidia and cystidia, if present, arise. The thickness of the fructification in such cases seems to be built up progressively by the activity of the surface layer. In this group also the basidia often arise directly from hyphal cells and then are commonly bifurcate at the base.

Even when restricted as indicated above the limits of the group are not yet clear. As here interpreted both cystidiate and gloeocystidiate forms would be included. A detailed study of several Bourdot and Galzin species that are not well known at present will need to be made as well as several described by Litschauer and others. A careful study of the method of proliferation should assist in determining the limits of the group. As a whole the group is difficult to study and close observation under the oil immersion lens is necessary.

The previously described species which would appear to be related as outlined above have been recognized for the most part by European students especially by Bourdot and Galzin and by Litschauer. In North America these forms have only recently been given attention. In Dr. Burt's treatment of *Corticium* (2) only one valid species was included—*C. pseudotsugae* Burt. Burt's *C. incanum*, as to **type** (cf. Rogers and Jackson, 8, p. 292) proves synonymous with *C. tulasnelloideum* Höhn. & Litsch. which in the meantime had been correctly recognized by Rogers (5) from Iowa. Rogers (6) has also described *C. subinvisibile* from Iowa and correctly recognized *Peniophora rimicola* (Karst.) Höhn. & Litsch. from Missouri. There are a few other European species which have been recognized from North American collections but have not yet been recorded. In addition to those described in this paper there are several undescribed species in herb. Rogers and in the University of Toronto herbarium.

Until the group is given further intensive study it cannot be stated with any certainty that all the species described below will prove to be related among themselves or to those discussed above. They are here presented for consideration by those who have the opportunity to study this group of delicate inconspicuous forms.

Description and Discussion of Species

Corticium inopinatum sp. nov. (Fig. 1)

Fructificatio effusa, delicata, tenuis, pruinosa, alba; subiculum macrum, hyphis 2–2.5 μ, irregulariter inflatis, nodoso-septatis; gloeocystidia abnormalia aut cylindracea, 20–60 × 5.5–6 μ, apice angusta, cylindracea, 4–6 μ longa projectura interdum praedito; basidia cylindracea vel clavata, 13–16 × 6–6.5 μ, quattuor sterigmatibus; basidiosporae globoideae, 4.5–6.5 μ diam., tunicis dense echinulatis, leviter incrassatis, non amyloideis.

Fructification effused in small areas, delicate, thin, pruinose, white or cream, forming a ceraceous film or when less well developed appearing like hoarfrost on the surface of substratum; subiculum meager, some hyphae 2–2.5 μ in diameter with clamps, cells often irregularly inflated, soon collapsing; gloeocystidia numerous, irregular, for the most part cylindrical, 20–60 × 5.5–6 μ, base sometimes bifurcate, apex occasionally with one or rarely two narrow cylindrical projections to 6μ long; basidia cylindrical or clavate, 13–16 × 6–6.5 μ, sometimes formed directly from hyphal cells and then bifurcate at base, bearing four slender, divergent sterigmata 5–6 μ long; basidiospores globoid with prominent basal apiculus, 4.5–6.5 μ in diameter including the markings, walls closely echinulate, slightly thickened, markings in optical section giving the appearance of a band 0.5–.75 μ broad surrounding the spore wall, non-amyloid.

Specimens examined:*

Ontario: On wood and bark of *Tsuga canadensis*, Strathgowan woods, North Toronto, Sept. 17, 1943, *18712, 18713, 19904* **type**, *19905*.

This gloeocystidiate species is separable because of the nature of the fructification and the unique character of the spores. Other gloeocystidiate forms with rough globose spores include *Gloeocystidium furfuraceum* (Bres.) Höhn. & Litsch., *G. analogum* B. & G., and *G. punctulatum* (Cke.) Lundell. The consistency of the fruiting body in all three species is very different from the one described above and in the species first named the spores are amyloid. There would appear to be no immediate relationship with any of them.

Though the spores are rough-walled, there does not seem to be any very close relationship with *C. tulasnelloideum* or with *C. insperatum* described below. The species seems unique amoug the forms having the general characters as outlined above.

Corticium insperatum sp. nov. (Fig. 2)

Fructificatio effusa, tenuis, subceracea, cinerea, adnata; subiculum paulum hyphis paralleliter currentibus diametri varri 1–2.5 μ nodoso-septatis, tunicis tandem gelatineis; basidia cylindracea vel subclavata, 8.5–13 × 4.5–6 μ, quattuor subulata sterigmata gerentia; basidiosporae ellipsoideae, 4–4.5 × 2.2–2.5 μ, tunicis dense minuteque asperulatis, non amyloideis.

* *Unless otherwise noted all collections were made by the writer. All are represented in the mycological herbarium of the University of Toronto, TRT.*

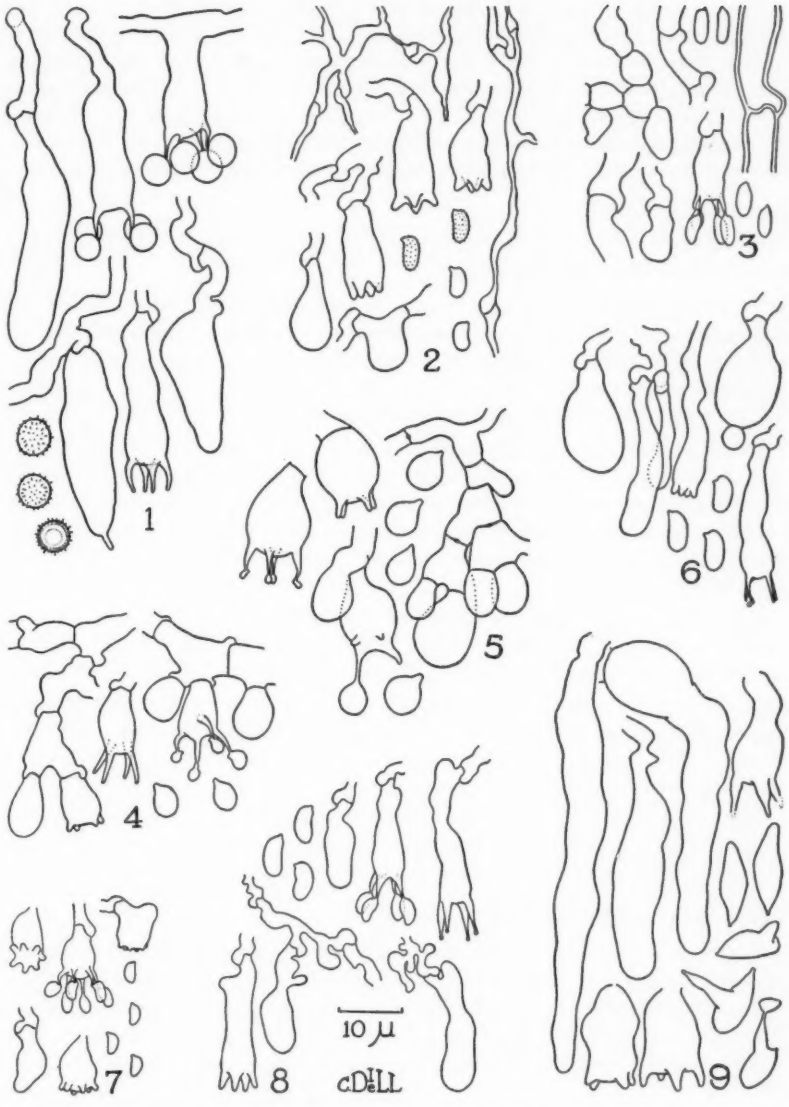

FIG. 1. *Corticium inopinatum*, gloeocystidia, basidia, and spores. FIG. 2. *C. inspersatum*, hyphae of subiculum, basidia, and spores. FIG. 3. *C. improvisum*, hyphae, basidia, and spores. FIG. 4. *C. exilis*, hyphae, basidia, and spores. FIG. 5. *C. permodicum*, hyphae, basidia, and spores. FIG. 6. *C. proximum*, gloeocystidia, basidia, and spores. FIG. 7. *C. delicatissimum*, basidia and spores. FIG. 8. *C. rallum*, basidia and spores. FIG. 9. *C. pauxillum*, gloeocystidia, basidia, and spores. *Reproduced at a magnification of approximately 1000×.*

Fructification effused, thin, subceraceous, waxy-pruinose, grayish or bluish-gray, adnate; subiculum scanty, made up below of more or less horizontal hyphae of irregular diameter 1–2.5 μ, with clamps, walls becoming gelatinized, subhymenium of intertwined, nodulose hyphae 1.5–2 μ in diameter; basidia cylindrical to subclavate, 8.5–13 × 4.5–6 μ, often bifurcate at base, bearing four subulate short sterigmata; basidiospores ellipsoid, 4–4.5 × 2.2–2.5 μ, straight on one side, walls closely and finely asperulate, nonamyloid.

Specimen examined:

Ontario: On bark of deciduous leaved tree, Timagami, Aug. 7, 1907, C. G. Lloyd BPI, *16008* **type.**

This species is obviously closely related to *Corticium tulasnelloideum* Höhn. & Litsch., first reported from North America by Rogers (5, p. 20) and frequently collected in recent years in Ontario and elsewhere. The latter has subgloboid spores somewhat variable in shape and size in different collections but not approaching the narrowly ellipsoid form of the species described above.

Bourdot and Galzin (1) included *C. tulasnelloideum* in their section Humicola of *Corticium*. It seems, however, to be quite unrelated to the more typical members of that section. They compared it with *C. submutabile* Höhn. & Litsch. Other rough-spored species included in that section which may on further study prove related are *C. ardosiacum* B. & G. and *C. stellulatum* B. & G.

Another related species is *Corticium tenuiculum* Litsch. described from a collection made in Sweden by L. Romell (Litschauer, 4, p. 130). In that species the spores are described as ellipsoid or obovate, 4–5 × 2.5–3 μ, and approach the obovate spores of some collections assigned to *C. tulasnelloideum*. Until more collections are available and further study has been made the number and validity of these rough-spored species which seem related to *C. tulasnelloideum* will perhaps remain in doubt.

It is possible that this group will be found to be related also to the *Peniophora rimicola* group as recently discussed by Jackson (3) though no capitate cystidioles have yet been observed in any collections examined.

Corticium improvisum sp. nov. (Fig. 3)

Fructificatio effusa, tenuis, cinerea membrana est; subiculum obscurum, paucis basalibus hyphis 4–4.5 μ diam., nodoso-septatis, subhymenio e brevibus, latis, abnormalibus ad 7 μ diam. cellulis composito; basidia cylindracea, 9–11 × 4–5 μ, quattuor subulata 3.5–4 μ longa sterigmata gerentia; basidiosporae cylindraceae, 4.5–5 × 2–2.5 μ, tunicis tenuibus, levibus, non amyloideis.

Fructification effused, thin, probably ceraceous when fresh, forming a grayish film on surface of substratum; subiculum obscure, a few basal hyphae 4–4.5 μ wide present, with clamps, occasionally broader and with thickened walls, subhymenium of short broad cells of irregular shape to 7 μ in diameter, giving a pseudoparenchymatous effect; basidia cylindrical, 9–14 × 4–5 μ,

with evident clamps at base, bearing four subulate, slightly arcuate sterigmata 3.5–4 μ long; basidiospores cylindrical, 4.5–5 × 2–2.5 μ, scarcely flattened laterally, walls thin, smooth, nonamyloid.

Specimens examined:

Quebec: On decorticated wood of *Quercus*, Morgan's woods near Macdonald College, Que., Aug. 27, 1941, **type**; Isle Perrot, near Montreal, Que., Aug. 27, 1941.

The above described species is distinguished by the pseudoparenchymatous subhymenium and the nearly cylindrical small spores.

Corticium exilis sp. nov. (Fig. 4)

Fructificatio effusa, tenuis, cinerea, vel ochracea; subiculum non conspicuum, paucis paralleliter currentibus 2–2.5 μ diam. hyphis, nodoso-septatis, subhymenio ex inflatis, abnormalibus 5–7 μ diam. cellulis composito; basidia cylindracea vel subclavata, 9–11 × 5–6.5 μ, quattuor subulata 6 μ longa sterigmata gerentia; basidiosporae obovatae, 4.5–5 × 2.5–3.5 μ, tunicis tenuibus, levibus, non amyloideis.

Fructification very thin, widely effused, gray to light ochraceous; subiculum not evident, a few horizontal hyphae 2–2.5 μ in diameter with clamps, from which arise branches composed of inflated and irregular thin-walled cells to 5–7 μ broad which give rise to the basidia; basidia cylindrical or subclavate, 9–11 × 5–6.5 μ, bearing four subulate divergent sterigmata to 6 μ long; basidiospores obovate, depressed on one side with prominent apiculus, 4.5–5 × 2.5–3.5 μ, walls thin, smooth, nonamyloid.

Specimen examined:

Ontario: On coniferous knot (*Tsuga* or *Pinus*), woods W. of Maple, Oct. 12, 1942, *18831* **type**.

This species appears to be distinctive because of the spore size and shape and the enlarged irregular cells from which the basidia arise. The relationship is not evident and it may not be closely related to any of the other species here described unless with the following species, *C. permodicum*, from which it differs in the smaller spores and basidia, and in the presence of clamps at the septa.

Corticium permodicum sp. nov. (Fig. 5)

Fructificatio paene visibilis, pruinosa, alba; subiculum e paucis paralleliter currentibus 3–3.5 μ diam. hyphis, non nodoso-septatis, compositum; basidia obovata, 12–15 × 7–8 μ, quattuor leviter divergentia 4–5 μ longa sterigmata gerentia; basidiosporae subgloboideae aut pyriformes, 6.5–7.5 × 5.5–6 μ, tunicis tenuibus, levibus, non amyloideis.

Fructification subinvisible, pruinose, appearing like hoarfrost on surface of substratum, white; subiculum practically nonexistent consisting of a few horizontal, simple-septate hyphae with thin walls 3–3.5 μ in diameter, from which short upright branches arise bearing the basidia; basidia obovate, 12–15 × 7–8 μ, the first ones terminal, proliferating from the cell below, bearing four

upright or slightly divergent sterigmata 4–5 μ long; basidiospores subgloboid or appearing pyriform when prominent basal apiculus is included, 6.5–7.5 × 5.5–6 μ, tending to cling together in groups of two or four, walls thin, smooth, nonamyloid.

Specimens examined:

Ontario: On wood of deciduous trees, Bear Island, Lake Timagami, Ont., July 18, 1936, *16704*, *16705* **type;** Aug. 8, 1937, A. J. Skolko *11962*.

The absence of clamps, larger spores, and basidia serve to distinguish this species from *C. exilis* with which it may possibly be related.

Corticium proximum sp. nov. (Fig. 6)

Fructificatio effusa, tenuis, alba, crustosa, in superficie minutis particulis tecta; subiculum ex hyphis 1.5–2 μ diam., nodoso-septatis compositum; gloeocystidia subvesiculosa, late clavata, 15–25 × 6.5–8.5 μ, tunicis tenuibus, apice globosa 3.5–4.5 μ diam. projectura interdum praedito; basidia clavata, 14–18 × 4–4.5 μ, quattuor gracilia recta sterigmata gerentia; basidiosporae, 4.7–5.5 × 2.5–3.5 μ, tunicis tenuibus, levibus, non amyloideis.

Fructification effused, thin, white or cream, when dry crustose, minutely granular; subiculum scanty, made up of thin-walled hyphae, 1.5–2 μ in diameter with clamps, which become somewhat torulose in the subhymenium; gloeocystidia subvesiculose, broadly clavate, 15–25 × 6.5–8.5 μ, walls thin, occasionally with a globose projection 3.5–4.5 μ in diameter at apex, rarely with two, not projecting appreciably; basidia narrowly clavate, 14–18×4–4.5 μ, bearing four slender, short, upright sterigmata 2.5–3.5 μ long; basidiospores ellipsoid, slightly depressed on one side, 4.7–5.5 × 2.5–3.5 μ, tending to cling together in groups of two-four, walls thin, smooth, nonamyloid.

Specimen examined:

Vermont: On frondose wood, Middlebury (E. A. Burt), **type,** FH.

This species is based on one of two collections made by Dr. E. A. Burt at Middlebury, Vt., which were listed as paratypes of *Corticium incanum* Burt, the type of which proves to be *Corticium tulasnelloideum* Höhn. & Litsch. (cf. Rogers and Jackson, 8, p. 292).

The relationship is with *Corticium albo-glaucum* Bourd. & Galz. from which it differs in the broader gloeocystidia and in the shape and size of the spores. In *C. albo-glaucum* the gloeocystidia (not previously described) are narrow, not over 4.5 μ and the apical projection, when present, is elongated rather than globose as in the species here described; and the spores are subnavicular or subfusiform, 7–9 × 2.5–3.5 μ.

Corticium delicatissimum sp. noc. (Fig. 7)

Fructificatio paene visibilis, pruinosa, alba; subiculum e paucis paralleliter currentibus nodulosis 2–2.5 μ diam. hyphis, nodoso-septatis, compositum;

basidia subglobosa, obovata aut pyriformia, 6–7 × 4.5–6.5 μ, 5–7 brevia, divergentia 2.5–3 μ longa sterigmata gerentia; basidiosporae ellipsoideae, 2.5–3.5 × 1.5–2 μ, tunicis tenuibus, levibus, non amyloideis.

Fructification subinvisible, pruinose, appearing like hoarfrost on surface of substratum, white; subiculum consisting of horizontal hyphae 2–2.5 μ in diameter, somewhat nodulose, with clamps, from which short branches arise which bear the basidia subtended by clamps; basidia, subglobose obovate or pyriform, 6–7 × 4.5–6.5 μ, bearing five–seven short divergent sterigmata 2.5–3 μ long; basidiospores ellipsoid, 2.5–3.5 × 1.5–2 μ, straight on one side with lateral apiculus, walls thin, smooth, nonamyloid.

Specimens examined:

Ontario: On rotting wood of *Tsuga canadensis*, woods W. of Maple, Ont. Sept. 11, 1943, *18721* **type,** *18836*.

C. delicatissimum is distinguishable by the five–seven minute spores borne on the basidia, and is not at all related to *C. suecicum* Litsch., or to *C. niveocremeum* Höhn. & Litsch., both of which also have nonurniform basidia bearing numerous sterigmata (cf. Rogers, 7, pp. 95–99). The relationship is rather with *C. subinvisabile* Rogers (6, p. 28) which is described as having six sterigmata and is assigned to Bourdot and Galzin's section Athele. The spores in that species are quite different and described as subfusiform, 6.5–7.5 × 2–3 μ, and the basidia correspondingly larger.

Corticium rallum sp. nov. (Fig. 8)

Fructificatio effusa, tenuis, subceracea, ceraceo-pruinosa, cinerea, adnata; subiculum infra obscurum, subhymenio ex intertextis nodulosis 1.5–2.5 μ diam., nodoso-septatis hyphis composito; basidia clavata vel subcylindracea, 10–16 × 4.5 μ, quattuor recta 4.5–5 μ longa sterigmata gerentia; basidiosporae, ellipsoideae, 5.5–6.5 × 2.5–3.5 μ, suballantoideae, tunicis tenuibus, levibus, infirme amyloideis.

Fructification widely effused, thin, subceraceous, waxy pruinose, grayish or bluish gray, adnate; subiculum obscure below, subhymenium made up of intertwined nodulose thin-walled hyphae 1.5–2.5 μ in diameter with clamps; basidia clavate or subcylindrical, 10–16 × 4–5 μ often bifurcate or inflated at one side, bearing four erect, slender sterigmata 4–5.5 μ long; basidiospores ellipsoid, 5.5–6.5 × 2.5–3.5 μ, straight or more commonly depressed on one side, suballantoid, rounded above and below, walls thin, smooth, weakly amyloid.

Specimens examined:

Ontario: On bark of *Tsuga canadensis*, woods W. of Maple, Nov. 22, 1941, *18717* **type;** on bark of *Acer* sp., woods S.E. of Aurora, Nov. 8, 1942, *17825*.

Iowa: On *Quercus macrocarpa*, W. Okoboji, Aug. 1, 1932, D. P. Rogers.

This is the only member of the section Athele which has so far been described as having amyloid spore walls. There are slight differences in the size of the basidia in the three specimens recorded but the spores are uniform and amyloid in all.

Corticium pauxillum sp. nov. (Fig. 9)

Fructificatio tenuis, delicata, effusa, pruinosa; subiculum obscurum, cellulis hypharum abnormalibus, collapsis, non nodoso-septatis; gloeocystidia cylindracea, omnem longitudinem extrudentia, 25–50 × 5–8 μ, tunicis tenuibus; basidia obovoidea vel late clavata, 13–20 × 6–7.5 μ, 3–4 fortia, divergentia sterigmata gerentia; basidiosporae ellipsoideae, 10–12 × 3.5–4.5 μ, repetitione germinantes; tunicis tenuibus, levibus, non amyloideis.

Fructification thin, delicate, effused, pruinose, when dry appearing under a lens to be made up of closely aggregated, white granules; subiculum scanty and obscure, cells of hyphae irregular, collapsed, without clamps; gloeocystidia cylindrical, sometimes slightly inflated below, extruded nearly their whole length, 25–50 × 5–8 μ, walls thin; basidia obovoid or broadly clavate, more or less cylindrical above, soon collapsing, 13–20 × 6–7.5 μ bearing three-four stout, divergent sterigmata; basidiospores ellipsoid or occasionally somewhat sigmoid, 10–12 × 3.5–4.5 μ, straight or slightly depressed on one side, germinating by repetition, with walls thin, smooth, nonamyloid.

Specimen examined:

Ontario: On rachis of *Pteretis pennsylvanica*, Bear Island, Lake Timagami; July 19, 1943, *18714* **type.**

This species appears to be quite unrelated to *Gloeocystidium cretatum* B.&G., which also occurs on ferns and the only one included in the Bourdot and Galzin section Athele of *Gloeocystidium*. This section is considered by them to correspond with the section of the same name in *Corticium*, but examination of authentic material of *G. cretatum* suggests that it is not closely related to the more typical members of the section Athele of *Corticium*.

Other than the species now included in *Ceratobasidium* Rogers and some of those included in *Pellicularia* Cooke, germination of the spores by repetition has been recorded in only one other species of the Homobasidiomycetes—*Peniophora heterobasidioides* Rogers—and that species may prove to be better placed in *Ceratobasidium*. It is possible that *P. pauxillum* could be included in *Ceratobasidium* also, but the few mature basidia seen do not have the extreme development of the sterigmata (epibasidia?) characteristic of that genus.

We have chosen to refer to the hymenial inclusions as gloeocystidia. Because of the extreme thinness of the fructifications they are extruded nearly their full length and could perhaps as well be called cystidia and the species described in *Peniophora*. These structures seem, however, to be more comparable to gloeocystidia. There is certainly no relationship to the more typical members of Bourdot and Galzin's section Gloeocystidiales of *Peniophora*, where the species would fall if described as a *Peniophora*.

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THE BLACK ROOTROT DISEASE OF TOBACCO.

II. PHYSIOLOGIC SPECIALIZATION OF *THIELAVIOPSIS BASICOLA* ON *NICOTIANA TABACUM*[1]

By R. H. Stover[2]

Abstract

Thielaviopsis basicola (Berk. & Br.) Ferraris exists in nature in two distinct forms, termed the brown and the gray wild type, which are differentiated on potato dextrose agar. Pathogenicity on tobacco was found to be a function of the wild type culture used. All gray wild type cultures were less pathogenic than the brown. The brown wild type cultures consisted of at least two physiologic races. Race I is found in the "old belt" of Ontario, and in Quebec, Ohio, and Connecticut. Race II is present in the "new belt" of Ontario and in Kentucky. Race II and all gray wild type cultures are less pathogenic than Race I. All cultural mutants were less pathogenic than wild type cultures.

Introduction

For a number of years it has been observed that certain tobacco varieties differ in their reaction to the black rootrot disease in different districts. For instance, certain varieties developed and distributed in the United States, primarily because of their resistance to black rootrot, proved to be susceptible when grown in infested soil at Harrow, Ont. With a view to obtaining an explanation of this phenomenon, pathogenicity experiments with different cultures of the causal fungus, *Thielaviopsis basicola* (Berk. & Br.) Ferraris, were planned. However, before they were undertaken, intensive cultural studies of the fungus were carried out, the results of which have been reported elsewhere (8). In these studies, it was shown that *T. basicola* may be present in soil or host in two different forms, termed the gray and the brown wild type, as differentiated on potato dextrose agar (P.D.A.). Both types were found to be coexistent in many soils, and the brown wild type, to be capable of mutating to the gray. Furthermore, a marked instability was found to characterize wild type cultures maintained on nutrient rich substrates. It may well be, therefore, that pathogenicity, as indicated by varietal response, is primarily a function of the wild type inoculum encountered in the geographic area where the test is made. Also, the results of pathogenicity experiments may be influenced to a considerable extent by the marked instability of wild type cultures maintained on artificial substrates. These two possibilities were brought to light through a detailed analysis (8) of the cultural behavior of *T. basicola*. Previous studies (1, 6) on physiologic specialization in this fungus failed to recognize or to consider them. It is the purpose of this paper

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to give an account of the investigations on the pathogenic behavior of the fungus in the light of these newly-acquired facts, as well as in relation to geographic areas.

Materials and Methods

During the summer of 1947, six preliminary experiments were undertaken to determine trends in pathogenic behavior, and to ascertain techniques upon which further studies could be based. These experiments were conducted with sterile and nonsterile soil, two varieties of tobacco, and one variety of watermelon.

It was found that brown wild type cultures from Harrow and Quebec were more pathogenic than gray type cultures from the same soils. The existence of physiological races was suggested by the reduced pathogenicity of brown wild type cultures from Delhi (Fig. 1), as compared with brown wild type cultures from Harrow or Quebec. Cultural mutants also were found to be less pathogenic than wild type cultures. It was evident that the results of these preliminary investigations were a function of the wild type cultures used, and that these varied with the areas from which the cultures originated. For example, brown wild types from Harrow and Quebec were more pathogenic than gray wild types from the same soils, yet these same brown types also were more pathogenic then brown wild types from Delhi soil. Therefore, it is not enough to differentiate between the brown and gray wild types, both of which may be present in the same soils: it is necessary also to recognize that from different geographic areas culturally similar but pathogenically distinct wild type races may be derived. Furthermore, variants arising in culture proved to be less pathogenic than the wild type cultures from which they were derived. As the wild types, and variants arising in culture, could be readily recognized on P.D.A. by their cultural characteristics, the majority of inocula were grown on this medium. Stock cultures were maintained in sterile sand or dried roots in order to prevent displacement of the wild type by cultural mutants. The cultural type was carefully recorded and cultural mutants were discarded from wild type cultures.

In addition to the influence of the cultural type on the pathogenic behavior of the various cultures, it was found that their pathogenic behavior was markedly affected by the amount of inoculum added to the soil. If the initial infestation was too heavy or too light, an accurate assessment of the pathogenicity of a culture was very difficult or impossible to make. For example, it was found that pouring inoculum over and about the roots usually resulted in uniformly severe disease on most varieties, regardless of the wild type cultures used. A determination of the optimum inoculum potential in the soil is, therefore, necessary before comparative experiments are undertaken. In addition to a proper amount of inoculum, light and temperature proved important in assessing pathogenicity. Thus, pathogenicity experiments cannot be satisfactorily carried out during the winter months, unless auxiliary lighting is used, or during July and part of August, when the temperatures are

too hot. The most satisfactory conditions for pathogenicity tests consisted of the following: (1) thorough mixing of the correct amount of inoculum through the soil; (2) the use of young, vigorous seedlings set in the soil one week after soil infestation; (3) and exposure to light approximating a minimum of a 12-hr. day of at least 600 ft-c.; (4) temperatures of from 18° to 25° C. Under such conditions, differences in pathogenicity were usually apparent after two weeks, and attempts were made in the experiments undertaken to approximate them as closely as possible. Soil pH, within the range six to seven and soil type were not found to exert a great influence on the results, provided the correct amount of inoculum was determined by preliminary experiments. To determine the correct balance of inoculum, it is necessary to carry out several trial experiments. Various proportions of soil and inoculum are thoroughly mixed and placed in small pots; these are planted with healthy seedlings, from 3 to 4 in. high, several days later. If the inoculum potential is not too heavy, differences in pathogenicity between different races is apparent after a period of from two to three weeks in both above and below ground symptoms. If the inoculum potential is too low, there may be no difference in pathogenicity evident above ground, although a careful inspection of the roots will usually show significant differences between races in respect to the number of small lesions present. If the light intensity is too low following planting, a retardation of plant growth occurs and hence greater injury is caused to varieties that are susceptible or moderately resistant to black rootrot than would normally occur under field conditions.

In the evaluation of the results of these pathogenicity experiments, it was finally decided to assign a "root index" to each infected root as follows: 100, no evidence of disease; 75, light infection; 50, moderate infection; 25, severe infection. Root index values are illustrated in Fig. 9.

All types of commercial tobacco varieties were used in the infection experiments, and the resistance of these to black rootrot in naturally-infested field plots at Harrow, and in greenhouse experiments, is listed in Table IV.

In Experiment 1, the pathogenicity of three cultural mutants (Table III) and a characteristic brown wild type culture was compared. One slant of each cultural type was added to a 4 in. pot of sterile loam, which was then planted with the variety Judy's Pride. The pathogenicity of several cultures from within each geographic area, as well as cultural variants, was compared in Experiment 2. These are described in Table I. Two to four cultures of each type, depending on the amount of growth, were added to each 4 in. pot of loamy field soil and one pot each of the following varieties were planted: White Mammoth, Judy's Pride, and Harrow Velvet.

Diseased root material and infested soils were collected from widely separated, representative tobacco-growing areas in Ontario and Quebec, as shown in Fig. 1. Isolates from this material, and from collections made in

TABLE I

DESCRIPTION AND SOURCE OF VARIOUS CULTURES USED IN INFECTION EXPERIMENT 2

Culture No.	Source	Cultural description
N	Newcastle	Monospore culture typical wild brown type
N_c	Newcastle	Cultural mutant mycelial tendency
N_s	Newcastle	Slight tendency towards submerged and mycelial growth
Q_g	Quebec flue-cured area	Monospore culture wild gray type
Q_2	Quebec flue-cured area	Brown type, appressed
Q	Quebec cigar area	Monospore brown type, woolly growth
Q	Quebec cigar area	Monospore brown type
H_g	Harrow	Wild gray type
H	Harrow	Wild brown type
V	Virginia	Wild gray type

FIG. 1. *Distribution of the Ontario and Quebec isolates of T. basicola used in pathogenicity studies, 1948. 1, Harrow; 2, Copenhagen; 3, Delhi; 4, Simcoe; 5, Alliston; 6, Newcastle; 7, Ottawa; 8, 10, Quebec cigar tobacco areas; 9, Quebec flue-cured tobacco area. The dotted line enclosing location 1 marks the "old belt" (Essex and Kent counties). Locations 2, 3, and 4 are included in the "new belt" (Elgin, Norfolk, Brant, and Oxford counties). Areas 5 and 6 have only been recently developed.*

Kentucky and Virginia, were used as the source of inocula in Experiments 3, 4, and 5; the data for these experiments are given in Table II. Unless otherwise stated, all isolates were of the brown wild type.

TABLE II

SUMMARY OF MATERIALS AND METHODS USED IN PATHOGENICITY TESTS WITH *T. basicola* ON DIFFERENT TOBACCO VARIETIES AT HARROW IN 1948

Expt. No.	Duration in days	Amount and type of inoculum*	Culture source	Soil type	Tobacco varieties
3	14	10-day old cultures on P.D.A. slants, 1 per 4 in. pot	Root isolations from 7 geographic areas (Fig. 3)	Sterile loam	White Mammoth Judy's Pride Harrow Velvet (seedlings)
4	45	10-day old cultures on P.D.A. slants, 10 slants per flat 24 in. × 12 in × 4 in.	Root isolations from 8 geographic areas (Fig. 4)	Loam	Seed of 8 varieties sown (Fig. 4)
5	40	14-day old 2% corn meal – sand cultures, 1 quart per flat 24 in. × 12 in. × 4 in.	Root isolations from 8 geographic areas (Fig. 5)	Loam	White Mammoth Judy's Pride Harrow Velvet (seedlings)

* *All cultures are of the brown wild type except the Virginia isolates, which are gray.*

Results of Pathogenicity Experiments

In Experiment 1 the pathogenicity of cultural mutants was determined in comparison to that of a wild type culture. The results summarized in Table III and Fig. 8 show that the cultural mutants were less pathogenic than the wild type culture.

TABLE III

A COMPARISON OF THE PATHOGENICITY OF THREE CULTURAL MUTANTS THAT ORIGINATED ON POTATO DEXTROSE AGAR SLANTS AND A WILD BROWN TYPE CULTURE

Culture description	Green weight in gm. of 4 plants (roots included)	Root index
Chlamydospore cultural mutant, endoconidia absent	75.7	80
Wisconsin cultural mutant, gray mycelial mat with chlamydospores and few endoconidia	75.5	80
Albino cultural mutant, complete absence of brown pigment	50.0	75
Wild brown type from Harrow	35.2	25
Check	75.0	90

These results are consistent with those obtained throughout the pathogenicity experiments. In all these experiments it was found that any variant arising in culture that differed significantly from the wild type in cultural behavior was reduced in pathogenicity. The greater the deviation in cultural characters of the progeny from a wild type culture, the greater was the reduction in pathogenicity. As a result of these observations, careful attention was given to the cultural behavior of a wild type culture before it was

used as a source of inoculum in comparative pathogenicity experiments. By discarding all cultures differing significantly from the parent wild type, the error caused by displacement of the original wild type by less pathogenic cultural mutants was minimized. No variant arising in culture was found to be more pathogenic than the wild type parent; even the albino type, which proved to be the most pathogenic of all cultural mutants, was considerably less pathogenic than any wild type.

The pathogenicity of cultures from different geographic areas, and cultural variants was compared in Experiment 2. The results of Experiment 2 are summarized in Fig. 2. The brown type cultures from Quebec and Harrow

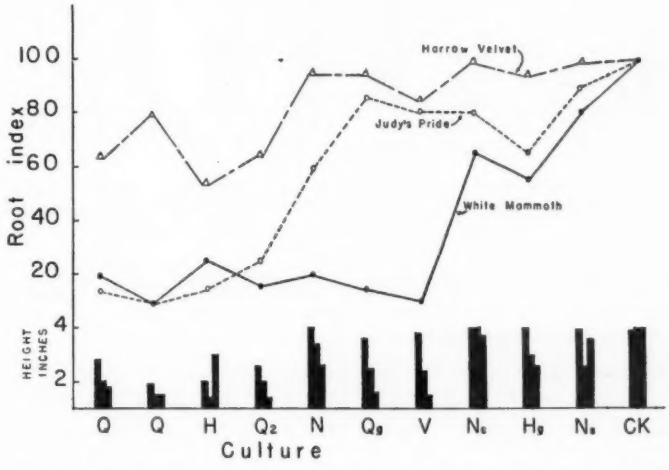

FIG. 2. *Results of Experiment 2. Cultures: Q, Quebec; H, Harrow; Q_2, Quebec flue-cured area; N, Newcastle (wild type); Qg, Quebec gray wild type; V, Virginia; Nc, Newcastle chlamydospore mutant; Hg, Harrow gray wild type; Ns, Newcastle submerged growth mutant; CK, check. Bars in shadowgrams from left to right represent the varieties Harrow Velvet, Judy's Pride, and White Mammoth.*

were much more pathogenic than the gray type cultures from the same areas or the brown type from Newcastle. The Harrow gray and brown wild types were from the same field, yet they display a marked difference in pathogenicity. The mutant cultures from the wild type Newcastle parent were less pathogenic than the Newcastle brown, wild type culture. The differential reaction of the tobacco varieties to the various strains is more evident in the resistant varieties than in the completely susceptible variety White Mammoth.

The pathogenicity of isolates from different tobacco-growing areas in Ontario and Quebec, and from Kentucky and Virginia, was compared in Experiment 3. The trend of the results in Experiment 3 (Fig. 3) is similar to that in Experiment 2 (Fig. 2). However, isolates from Ottawa, Delhi, and Kentucky are included. The culture from Wisconsin was dominated by mycelial mutants

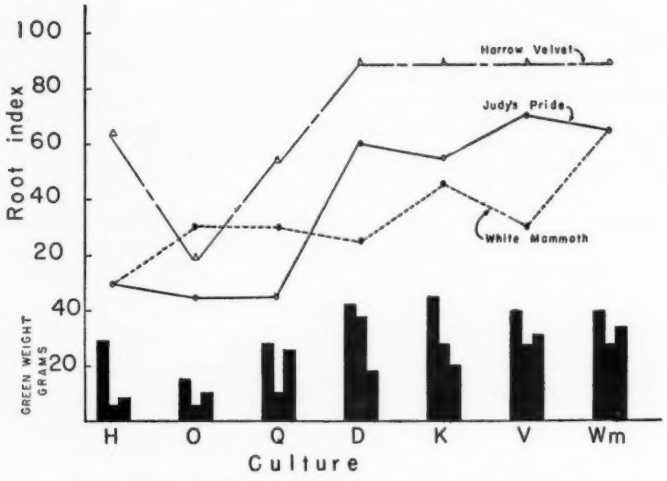

FIG. 3. *Results of Experiment 3. Culture: H, Harrow; O, Ottawa; Q, Quebec; D, Delhi; K, Kentucky; V, Virginia; Wm, Wisconsin mutant. The bars in each shadowgram represent from left to right the varieties Harrow Velvet, Judy's Pride, and White Mammoth.*

and was only slightly pathogenic. The pathogenicity of the Delhi, Kentucky, and Virginia isolates is less than that of the Harrow, Ottawa, and Quebec cultures. These results are illustrated in Fig. 6.

The reaction of nine different varieties and types of tobacco to eight different geographical isolates of *Thielaviopsis basicola* was determined in Experiment 4. Fig. 4 shows, in terms of the root index system, the greater pathogenicity of

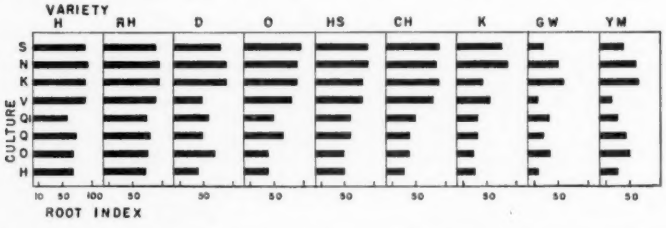

FIG. 4. *Results of Experiment 4. Variety: H, Harmony; RH, Resistant Havana 211; D, Delcrest; O, Oxford 400; HS, Halley's Special; CH, Connecticut Havana 38; K, Kelley; GW, Greenwood; YM, Yellow Mammoth. Resistance in the field decreases from Harmony to Yellow Mammoth (see Table IV). Culture: S, Simcoe; N, Newcastle; K, Kentucky; V, Virginia; Q_1, Quebec flue-cured area; Q, Quebec; O, Ottawa; H, Harrow.*

the Quebec, Ottawa, and Harrow isolates on the more resistant varieties Harmony, Resistant Havana 211, Delcrest, Oxford 400, Halley's Special, and Connecticut Havana 38, as compared with the isolates from Simcoe, Newcastle, Kentucky, and Virginia. As the varieties decrease in resistance,

differences in pathogenicity between isolates decrease. Even in very susceptible varieties, however, a slight differential is present. These results are illustrated in Fig. 7.

The pathogenicity of geographically different isolates of *T. basicola* growing in corn meal – sand was determined in Experiment 5. This experiment (Figs. 5

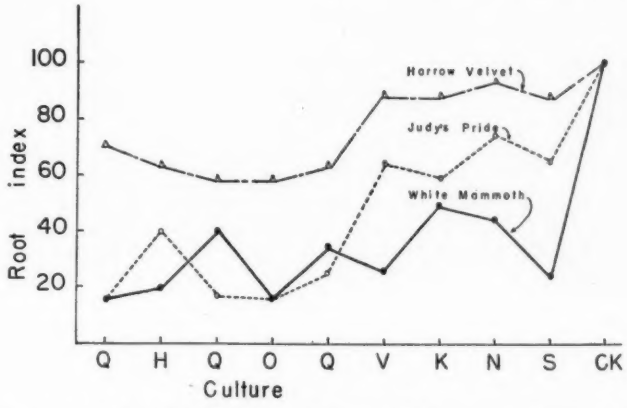

FIG. 5. *Results of Experiment 5. Culture: Q, Quebec; H, Harrow; O, Ottawa; V, Virginia; K, Kentucky; N, Newcastle; S, Simcoe; CK, check.*

and 9) clearly illustrates the differences in pathogenic behavior of culturally indistinguishable wild type cultures of *T. basicola* from different geographic areas, as indicated by the reaction of three tobacco varieties. The variety White Mammoth is classified in field tests as completely susceptible to black rootrot in Canada and it is susceptible to all isolates in this experiment.

SUMMARY OF RESULTS OF EXPERIMENTS 1 TO 5

From the results of these experiments, it is evident that *T. basicola* consists of different physiological races. The various isolates used may be grouped according to their pathogenic behavior as follows:

Race I—brown wild type cultures, represented by isolates from Harrow, Ottawa, Quebec, Ohio,* and Connecticut.*

Race II—brown wild type cultures, represented by isolates from Kentucky, Delhi, Newcastle, Simcoe, Copenhagen (Ont.),* and Alliston.*

Race III—all gray wild type cultures from the areas representing Race I.

Race IV—gray wild type cultures from Virginia, the only area in which brown wild type cultures have not been found thus far.

In Table IV, the reaction of different tobacco varieties to these physiological races is shown and compared with the reaction of these varieties in rootrot

* *Pathogenicity experiments with isolates from these areas were carried out during the summer of 1949. See Fig. 1 for the location of the areas named.*

TABLE IV

A COMPARISON OF THE RESISTANCE OF TOBACCO VARIETIES IN GREENHOUSE TESTS TO FOUR DIFFERENT PHYSIOLOGICAL RACES AND IN NATURALLY-INFESTED HARROW SOIL

Variety	Varietal reaction		
	To races II, III, IV	To race I	In naturally-infested field soil at Harrow
Harrow Velvet*	90	75	85**
Resistant Havana 211	90	80	85
Harmony	90	70	75
Delcrest	80	55	65
Oxford 400***	80	50	50
Connecticut Havana 38	85	40	50
Halley's Special	75	30	45
Judy's Pride	65	30	40
Yellow Mammoth	50	25	30
Greenwood	40	20	30
White Mammoth	30	20	20
Kelley	50	30	20

** The varieties are arbitrarily subdivided into groups of increasing susceptibility: highly resistant, moderately resistant, slightly resistant, moderately susceptible, and completely susceptible.*

*** Root index (see p. 728) figures are composite, representing data from the rootrot plots at Harrow and greenhouse experiments.*

**** This variety is classified as resistant to black rootrot at Oxford, N.C.*

plots at Harrow. The varieties may be classified arbitrarily as to their resistance and susceptibility, as indicated by their reaction to the disease in naturally infested soils at Harrow. As shown by the root index, the reaction of varieties to infection by race I isolates is similar to their reaction in naturally-infested soil at Harrow. With isolates of races II, III, and IV, the reaction is different; the varieties manifesting moderate and slight resistance to race I react as highly resistant to races II, III, and IV. Only a few race III cultures were included in these experiments and all were found to be somewhat less pathogenic than their brown counterparts. Varieties that are moderately and completely susceptible to race I are moderately resistant and moderately susceptible to races II, III, and IV.

Gray wild type cultures were not sufficiently representative in these experiments to make a comparative analysis of their pathogenicity. It is known that gray wild type cultures are considerably less pathogenic than the brown

FIG. 6. *Experiment 3—Variety Judy's Pride (moderately susceptible) inoculated with the following isolates: left to right, Check; Quebec; Harrow; Ottawa; Delhi; Virginia; Kentucky; Kentucky 2.*

FIG. 7. *Experiment 4—Variety Harmony infected with cultures from the following areas: A, Quebec; B, Ottawa; C, Harrow; D, Kentucky; E, Virginia; F, Simcoe; G, Newcastle.*

PLATE I

6

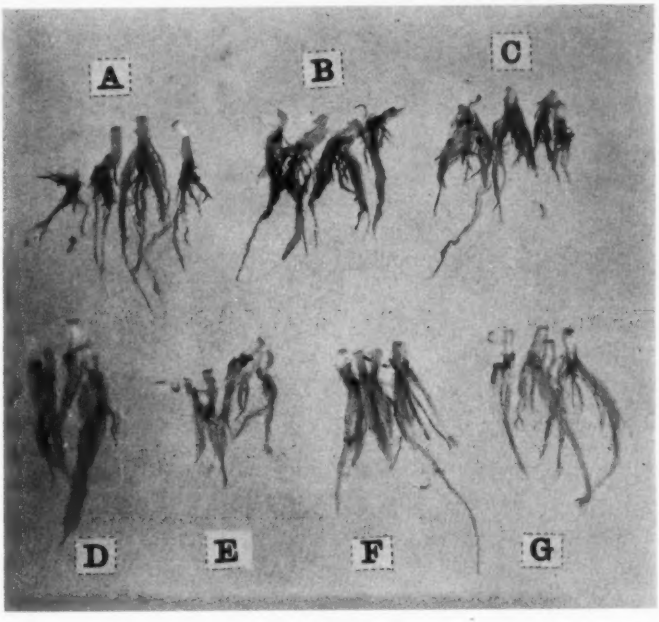

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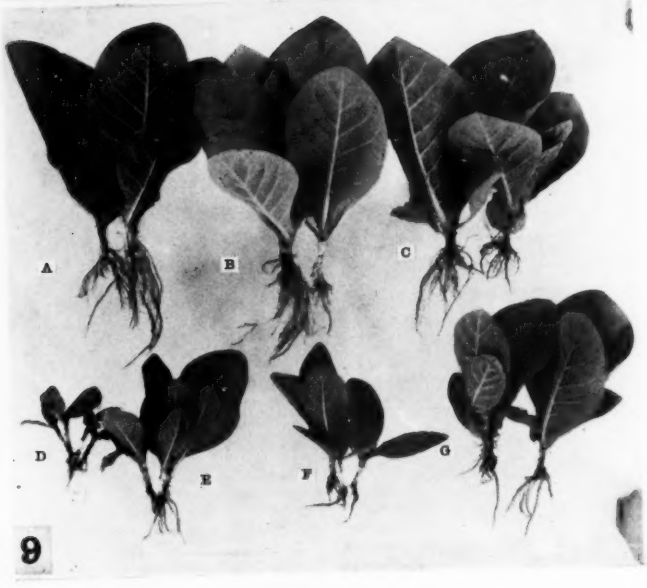
PLATE II

8



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wild type representing race I (cf. Fig. 2). However, the gray wild types from race II areas have not been included in sufficient numbers to determine their pathogenicity, although the results of preliminary experiments indicate that these also are less pathogenic than their brown wild type counterpart. A comparison, therefore, needs to be made of the pathogenicity of the gray wild type from areas representing races I, II, and IV.

Recent experiments with brown and gray wild types that were maintained in artificial culture for from one to several years indicated that changes in the inherent pathogenicity of the original isolate may occur during the period in culture. Such changes were most evident in species other than tobacco, which included beans, soybeans, peas, watermelon, red clover, sweet clover, and alfalfa. The nature of these changes in pathogenicity, within the brown and gray wild types, is not well understood. More extensive tests are needed to determine the pattern of changes in the pathogenic capabilities of the fungus that may occur in artificial culture and in the host.

Discussion

Allison (1) found that isolates of *T. basicola* from the northern United States were more pathogenic than those from the southern towards several tobacco varieties. However, he did not recognize the existence of different wild types. In the present studies, these findings were found to hold when certain north and south races were compared, but not with others. Thus, when gray wild type cultures from Canada or Ohio are compared with cultures from the Southern United States (Virginia and Kentucky), the differences in pathogenicity to tobacco are not significant. Also, brown wild type cultures from within the "new belt" area in Ontario, and from more recently developed areas (Fig. 1) over 100 miles north and east of the "new belt", are of the same pathogenic order as the isolates from the southern United States. All of these brown wild type isolates have proved to be less pathogenic than brown wild type cultures from the "old belt" in Ontario, and from Quebec, Ohio, and Connecticut. Furthermore, the importance of using wild type cultures only and the necessity of eliminating cultural variants in all comparative wild type pathogenicity experiments are very evident.

The characteristics of the two distinctly different tobacco-growing areas in Ontario suggest several hypotheses to account for the difference between the two areas in respect to the pathogenic capabilities of *T. basicola*. The more

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FIG. 8. *Experiment 1—Comparison of the pathogenicity of three cultural mutants with a typical wild type culture of T. basicola: top right, check; top left, chlamydospore mutant (endoconidia absent); bottom right, Wisconsin mutant (mostly mycelium and chlamydospores); bottom left, albino; center, brown wild type. Few lesions were present on the roots of the plants from mutant cultures. The albino culture was the most pathogenic of the mutants.*

FIG. 9. *Experiment 5—Variety Judy's Pride infected with cultures from the following areas: A, Newcastle; B, Simcoe; C, Virginia; D, Quebec (flue-cured area); E, Quebec; F, Ottawa; G, Harrow. A root index would be assigned as follows: A, 75; B, 70; C, 65; D, 10; E, 25; F, 15; G, 30.*

recently developed tobacco-growing districts, including the "new belt", have been under cultivation with this crop no longer than 20 years, and in many locations less than 10 years. In contrast, tobacco has been grown in the "old belt" in Ontario, and also in Quebec, for at least 75 years. The recently developed Alliston and Newcastle areas (Fig. 1) comprise virgin tobacco land situated over 100 miles from the "old belt". Nevertheless, *T. basicola* was prevalent in many of these fields after as few as three consecutive crops of tobacco had been grown in them. Therefore, cultures from these areas are probably closely representative of the fungus as it exists as a natural soil habitant before a highly parasitic existence was made possible by the continued presence of a highly susceptible host. These cultures are similar in pathogenicity to those from the "new belt", both being less pathogenic than the "old belt" cultures. Although little is known about the behavior of the fungus in soil in the absence of a susceptible host, it may be assumed that it exists as a weak parasite and as a saprophyte, but does so only at a low level of subsistence. Incipient lesions with a few chlamydospores have been found occasionally on oats and rye roots growing in infested soil. It may be that the fungus exists on the roots of native plants as a weak parasite until crops more susceptible to attack are grown, in a manner somewhat similar to that of *Ophiobolus graminis* (5). If a weakly parasitic existence has been maintained on native plants for an indefinite period in areas where resistant or slightly susceptible hosts are present, then it is not unreasonable to suppose that, with the introduction and continued cultivation of a highly susceptible host, the fungus might become increasingly virulent toward that host with the passage of time. If such were the case, the fungus in the older tobacco-growing areas should have an advantage in parasitism over that in the "new belt" area.

T. basicola was reported as causing a disease of lupine in Germany 60 years ago (10), and has subsequently been reported as attacking various legume species in Europe. Why the fungus has not been reported more generally in Canada and the United States on clovers, beans, and other legumes in tobacco-growing districts, and in areas removed from tobacco belts, is not known. It is probable that the fungus is more common on these hosts than is suspected, as it has been recently reported as causing a disease of lima beans in California (7) and one of watermelons in Utah (4). Yarwood (9) has found the fungus in California soils where crops that are not attacked were grown. Hildebrand and West (2) have found *T. basicola* to be prevalent in Ontario on soybeans grown under certain conditions. The writer has found that the brown wild type strains from tobacco readily attack watermelon, sweet clover, alfalfa, and white clover in artificially-infested soils. Cultures of *T. basicola* have been isolated on potato discs from field crops of sweet clover, red clover, soybeans, white beans, and alfalfa. It may be that continuous or frequent culturing on certain hosts is necessary to the evolution and selection of strains highly pathogenic to them. With the continuous cropping of legumes in the

same soil, strains of the fungus highly virulent for legumes in the area concerned may develop. Further studies are needed to determine the relationship of the tobacco strains to legume hosts. This relationship could most readily be ascertained by growing various legumes in soils known to be heavily infested with the tobacco strains of *T. basicola*. In Ontario, susceptible varieties of dark tobacco are sometimes severely injured by black rootrot in fields where tobacco has not been grown for from 8 to 20 years. In these fields legumes form a part of the rotation system.

It is possible also that the introduction of a resistant host might induce a change in pathogenicity, following the build-up of a certain inoculum potential through the use of susceptible hosts. Resistant tobacco varieties have been grown in the "old belt" of Ontario and in Quebec for 20 years, whereas the varieties grown in the "new belt" have been completely susceptible to attack by *T. basicola* until the recent introduction of a resistant flue-cured type. Evidence has already been given (8) that the host probably does exert an effect on the mutation of the wild types of the fungus. This effect was indicated in experiments in which the gray-to-brown mutation was found to be associated only with host passage. These brown type cultures, derived from the gray by mutation, have not yet been examined for their pathogenic behavior. However, since the brown types that have been tested have always been found to be more pathogenic than their gray counterparts, it is probable that these derived brown cultures will be more pathogenic than their gray parent. Such a finding would indicate that a less virulent gray wild type has mutated to a more virulent brown. The next step in the evolution of virulence would be the derivation from race II "new belt" cultures of a wild brown type of the same pathogenic capabilities as race I "old belt" isolates. Further intensive host passage, cultural, and pathogenicity studies are necessary to test this hypothesis.

An apparent objection to the above theory is the difficulty of explaining the less virulent behavior of cultures from the southern United States. One of the more fundamental differences, however, between the areas of tobacco culture in Canada and the southern United States is the climatic one. Of the several factors affecting the pathogenicity of *T. basicola*, soil temperature has been found to be the most decisive (3). In the states to the south of North Carolina, Virginia, and Kentucky, where soil temperatures during the critical growing period are high, black rootrot is not a serious disease problem, whereas in the more northerly states it can cause severe injury. It may be that the inherent pathogenic capabilities of the fungus are influenced or dominated by the effects of high soil temperature in areas such as Virginia and Kentucky. In this respect, a comparison of soil temperatures during the critical growing period in the areas concerned would be of considerable value. The high summer temperatures may also exert a selective effect on the survival of fungus strains from one season to the next. In addition to temperature, soil types and rotations may be of considerable importance, particularly as they influence the

soil flora. In any case, the evolution of fungus virulence from the early inception of the tobacco industry in these areas would be subjected to the selective action of a soil–host–environment complex differing significantly from that in more northerly states or Canada.

Acknowledgments

The writer gratefully acknowledges the generous advice and assistance given by Prof. D. L. Bailey throughout the course of these studies. Thanks are due to Dr. L. W. Koch for valuable assistance and encouragement, and for rendering available the facilities of the Dominion Laboratory of Plant Pathology, Harrow, Ont. For financial assistance and continued interest, the author is grateful to The Imperial Tobacco Company of Canada, Limited. To Mr. R. J. Haslam, of the Experimental Station, Harrow, Ont., and to the Officer-in-Charge of the Experimental Substation, Delhi, Ont., the writer wishes to express his gratitude for providing materials and field facilities. The co-operation of many individuals in Canada and the United States in providing samples of soil and plant material is gratefully acknowledged. Thanks are due to Dr. R. G. Henderson of Blacksburg, Va., for providing numerous specimens.

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AN AGAR-SHEET METHOD OF TESTING THE EFFICIENCY OF SEED TREATING MACHINES[1]

By J. E. Machacek[2]

Abstract

The efficiency of a seed treating machine depends to a considerable extent on the uniformity with which the fungicide used for treatment is mixed with the seed. The uniformity of treatment may be ascertained by placing treated seeds, at 2-in. intervals, on large sheets of inoculated potato-sucrose agar. In the preparation of these sheets, melted, acidified agar is poured in a uniform layer over panes of glass and, when the agar has solidified, it is dusted heavily with spores of *Penicillium purpurogenum* Stoll. After the treated seed is placed on the agar, the culture is covered with a sheet of glass and incubated for two days at room temperature. The uniformity of treatment may then be determined from the variability in diameter of the zones of inhibition around the seeds. This technique may also be used for determining the comparative potency of different fungicides, and the rate at which they are lost from the seed.

Introduction

A simple laboratory method of determining the variability in the load of fungicide on treated seed has been developed by the author. By this method, which is a modification of one described by Mead (2), a number of treated seeds are plated on a large sheet of agar inoculated with fungous spores, and the variability in load of fungicide is then determined after a measurement of the diameters of the zones of inhibition around the seeds has been made.

From observation, this plating method may be used for other purposes, e.g., bioassay of fungicides, determining the rate of loss of fungicide from seed, and demonstration of the effect of seed treatment. A description of the method follows, although the investigation for which it was originally developed has not yet been completed.

Procedure

Before this plating method can be used, it is necessary to prepare a supply of shallow sheet-glass trays of a predetermined size. For general use, the author prefers to make them from "double diamond" panes of greenhouse glass (16 in. × 24 in. × 1/8 in.). Along the margin of each glass sheet is affixed a frame of glass strips, 1/2 in. wide and 1/8 in. thick. These glass strips are cut in two lengths, 16 in. and 23 in., and, at the time they are cut, some allowance should be made for breakage, and an additional allowance (two extra strips for each agar container made) for a purpose to be mentioned later. The frame of glass strips is attached to the glass pane with a suitable adhesive, such as Miracle Adhesive. On the following day, the completed trays are washed and dried. They are then ready for use.

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Contribution No. 1045 from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Canada.

[2] *Plant Pathologist.*

When about to be used, the trays are placed on a flat surface and levelled individually with a spirit level small enough to fit within the frame. Strips of paper may be inserted under any tray where it is too low. This levelling is essential as, without it, the depth of the sheet of agar in the tray will not be uniform and this affects the readings subsequently taken.

After the trays are levelled, they are given a preliminary warming by wiping them over twice with a cloth moistened with hot water. The purpose of this warming is to reduce the danger of breakage of the tray when melted agar is poured into it.

Into each tray is poured 500 cc. of melted potato-sucrose agar, containing 2% sucrose, 1.2% agar, and acidified with 85% lactic acid at the rate of 1 cc. acid to 500 cc. agar. The pouring should be done quickly and in a zigzag fashion over the bottom of the tray, care being taken that the corners are filled. If bubbles occur on the surface of the agar, they may be removed by suction with a pipette.

After the agar in the trays has become solid, a cloud of spores of the test fungus is blown over it from a Petri dish or flask culture. In actual practice, there is little residual contamination of the laboratory. *Penicillium purpurogenum* Stoll has been found to be a very satisfactory fungus for the present purpose, as it produces an abundance of dry spores in a comparatively short time and is also sensitive to a number of fungicides. Cultures of this fungus, when grown in Erlenmeyer flasks containing ground oats moistened with a 0.5% solution of peptone in water, were even more sporiferous than cultures on potato-sucrose agar in Petri dishes. An atomizer bulb with a 6-in. rubber extension tube aids in the blowing of spores from either type of culture.

When testing the distribution of a fungicide on seeds, the inoculated agar trays are placed on a seeding-pattern plate marked with pencil or ink at points above which the individual seeds are to be put on the agar. Such a plate may be made by inserting a sheet of squared paper, marked as required, between two sheets of glass held together at the edges with a strip of adhesive tape (a 2-in. rectangular spacing, allowing 77 seeds to an agar sheet, is preferred by the author). Treated seeds are taken individually from a treated lot and placed on the agar according to pattern. After the seeding of a sheet of agar is completed, a pane of glass equal in size to that of the tray is laid on the seeds, thus pressing them partly into the agar. The sheet of glass is then raised slightly and two strips of glass (some of the extras mentioned earlier) are inserted between it and the edges of the tray on two opposite sides. This allows aeration of the culture without undue desiccation of the agar. To conserve space, seeded trays of agar with their covering glass sheets may be stacked on any convenient flat surface.

The temperature of the room used for incubation of the seed cultures should not be allowed to vary to any great extent, lest drops of condensed moisture form on the undersurface of the covering sheets and fall on the treated seeds. If the temperature is held between 20°–24° C., a dense felt of

fungus growth, with the exception of clear, circular zones of inhibition around treated seeds, covers the inoculated agar sheet within 48 hr. The diameters of these clear zones may be measured with a rule marked off in millimeters, the process of measurement being greatly expedited if one person takes the measurements and another records them.

The variability in diameter of the zones of inhibition (indicating dispersability, or degree of dispersion for a fungicide) may be determined by the formula

$$\text{Variability} = \Sigma X^2 - T^2/N$$

where ΣX^2 is the sum of the squares of individual measurements and T^2/N is the squared total of measurements divided by their number. The mean of the measurements can be used as an index of fungicide potency or toxicity. If there are a number of replicate sowings, and several variables (different fungicides, different dosages, etc.) are included, the data may be analyzed statistically in the usual way.

Some Results Obtained by the Agar-sheet Method

The Dispersability of Fungicides on Seed

Two dusts, one slurry, and a fluid with an oil base (A, B, C, and D, respectively) were applied to wheat seed at the rate recommended by their manufacturers. Each mixture was given 200 agitations in a laboratory shaker designed and made by Mr. R. H. Cunningham of the Dominion Laboratory of Cereal Breeding, Winnipeg, Man. (1). The data obtained by plating this seed, summarized to show the variability in diameter and the mean diameter of the zones of inhibition for each of 16 sheets of agar, each sown with 77 treated seeds, are given in Table I. The agar sheets from one replicate are illustrated in Fig. 1.

The data on variability—a low figure indicates uniform dispersion— in Table I indicate that the dusts (A and B) and the slurry (C) became dispersed fairly uniformly on the treated seed. On the other hand, fungicide D did not disperse very well (compare A and D, Fig. 1). The potency of fungicides A and C appears to be greater than that of fungicides B and D. Thus potency does not appear to be related to dispersability.

A study was made also of the degree of dispersion attained by fungicide A when applied to wheat seed at various rates (1/4, 1/2, 3/4, and 1 oz. per bushel) and the treated seed was given 50, 100, 150, or 200 agitations in the laboratory shaker. The means of the data on variability from four replicates of the test show (Table II) that for fungicide A (a dust) a better mixture was obtained after 150 agitations than after 50 or a 100, and that the amount of the fungicide (above 1/4 oz. per bu.) in this case had comparatively little to do with the number of agitations required to achieve homogeneity in the mixture. The number of agitations had no effect on the mean diameter of the zone of inhibition, but the amount of fungicide had—the larger the amount the larger the diameter.

TABLE I

THE DISPERSABILITY AND APPARENT POTENCY OF FOUR FUNGICIDES APPLIED TO WHEAT SEED

Property studied	Fungicide	Replicate				Mean
		I	II	III	IV	
Dispersability (variability in diameter of zones of inhibition)	A	10.26	5.43	3.32	5.42	6.11
	B	7.09	7.23	10.30	4.71	7.33
	C	9.82	13.09	10.39	3.05	9.08
	D	27.04	33.80	15.02	30.91	26.69
	Mean	13.55	14.89	9.76	11.02	
	Sign. diff. (5%)	D.N.S.*				7.19
Potency (diameter of zones of inhibition, cm.)	A	1.971	2.515	2.411	2.605	2.375
	B	1.298	0.967	1.200	1.444	1.227
	C	2.828	2.689	2.833	2.931	2.820
	D	1.323	1.654	1.277	1.703	1.457
	Mean	1.855	1.956	1.930	2.171	
	Sign. diff. (5%)	D.N.S.*				0.262

* *D.N.S. = Difference not significant.*

In another test, the fluid fungicide D was applied to wheat seed at 1/4, 1/2, 3/4, and 1 fl. oz. per bushel and each mixture was agitated 200 times. The results from four replicates of this test (Table III) show that this fungicide did not disperse as uniformly as did the dust fungicide A (Table II), and that the amount applied to the seed had no effect on the uniformity of the mix.

Demonstration of Inactivation or Loss of Fungicide on Treated Seed

One-pound lots of wheat seed were treated, at the dosages normally used, with four fungicides, A, B, D, and E. As mentioned before, fungicides A and B were dusts and fungicide D was dissolved in an oil carrier. Fungicide E was a fluid with an alcohol base. Each mixture was given 200 agitations in the laboratory shaker and then was divided into two parts, one part being kept in a sealed glass container and the other part being spread out in a thin layer in an open, shallow glass dish. Nineteen seeds from each treatment and the same number from the untreated check were placed in rows (three seeds to an inch) on a sheet of inoculated agar immediately after treatment. Other platings of the seed kept in open dishes were made at weekly intervals. After six weeks, a plating was made also of the seed kept sealed in the glass container. Illustrations of the plates when sown with newly-treated seed, with treated seed held at room temperature for three and six weeks in open dishes,

PLATE I

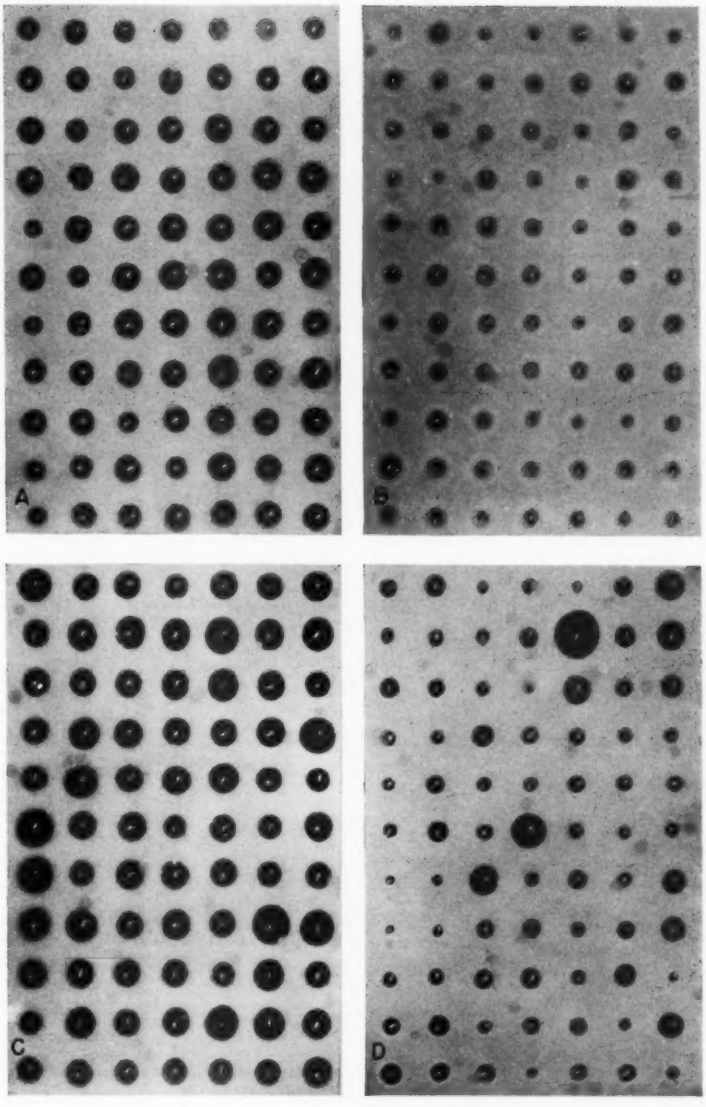

FIG. 1. *Dispersability of fungicides on wheat seed: A, fungicide A applied as a dust; B, fungicide B applied as a dust; C, fungicide C applied as water slurry; D, fungicide D applied in oily carrier.*

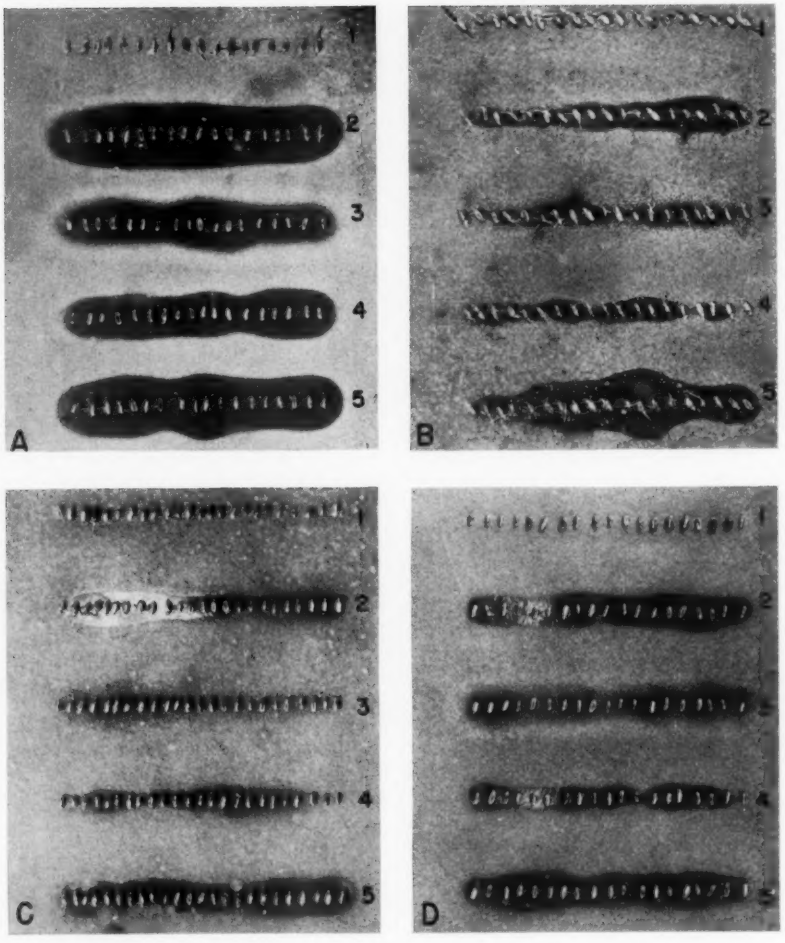

FIG. 2. *Rate of loss of different fungicides from treated seed held in storage at 65°–75° F.: A, seed plated immediately after treatment; B, seed exposed to air for three weeks; C, seed exposed to air for six weeks; D, seed kept in sealed glass jar for six weeks. From top to bottom: 1, untreated seed; 2, fungicide A (dust); 3, fungicide B (dust); 4, fungicide E (water-miscible carrier); 5, fungicide D (oily carrier).*

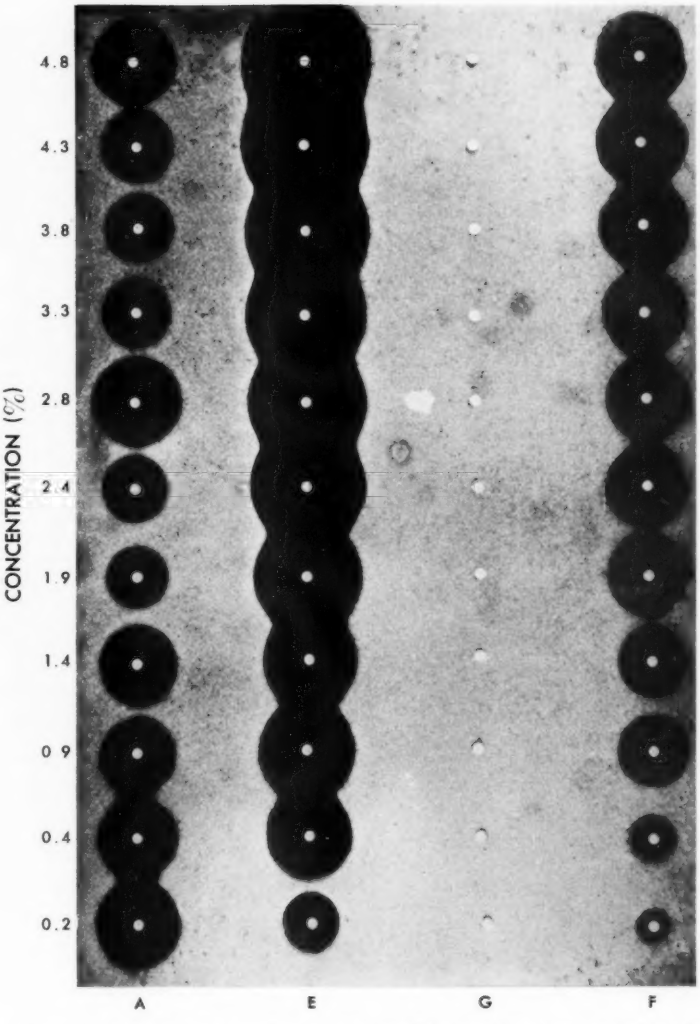

FIG. 3. *Diffusion into inoculated agar of fungicides from filter paper soaked in solutions of different concentrations; A, fungicide A; E, fungicide E; G, fungicide G (no inhibition of fungus); F, fungicide F.*

TABLE II

EFFECT OF DOSAGE AND NUMBER OF AGITATIONS GIVEN TREATED WHEAT SEED ON THE DEGREE OF DISPERSION OF DUST FUNGICIDE A AND ON THE DIAMETER OF THE ZONE OF INHIBITION

Effect studied	Dosage (oz./bu.)	No. of agitations				Mean
		50	100	150	200	
Dispersion	0.25	21.91	12.84	8.26	10.22	12.78
	0.50	13.18	7.38	7.08	5.31	7.40
	0.75	7.15	4.05	5.09	4.37	6.30
	1.00	8.89	5.34	4.77	4.01	5.97
	Mean	13.30	8.24	5.16	5.75	
	Sign. diff. (5%)	3.07				3.79
Diameter of zone of inhibition, cm.	0.25	1.364	1.691	1.633	1.796	1.621
	0.50	2.472	2.590	2.377	2.611	2.513
	0.75	2.945	3.310	3.046	2.968	3.067
	1.00	3.392	3.304	3.394	3.495	3.396
	Mean	2.543	2.724	2.612	2.717	
	Sign. diff. (5%)	D.N.S.*				0.364

* *D.N.S. = Difference not significant.*

TABLE III

EFFECT OF VARYING DOSAGE ON THE DEGREE OF DISPERSION OF FLUID FUNGICIDE D ON TREATED WHEAT SEED

Effect studied	Dosage (fl. oz./bu.)	Replicate				Mean
		I	II	III	IV	
Dispersion	0.25	94.06	4.99	14.76	16.50	32.57
	0.50	63.44	34.46	22.46	19.74	35.02
	0.75	29.78	48.14	16.75	21.01	28.92
	1.00	39.01	37.90	19.57	31.53	32.00
	Mean	56.56	31.37	18.38	22.19	
	Sign. diff. (5%)	D.N..S*				D.N.S.*

* *D.N.S. = Difference not significant.*

and with treated seed held at room temperature in closed containers for six weeks are given in Fig. 2. From the appearance of the zones of inhibition, it would appear that fungicides A and D were retained fairly well on treated

seeds, whereas fungicides B and E were not. It is worthy of note that storage, even in a sealed container, did not prevent some loss of the fungicidal principle added to the seed.

Determining Special Characteristics of Fungicides

Fungicides A, B, E, F, G, and H were added to water to make up a series of concentrations ranging from a maximum of 4.8% to a minimum of 0.2%. Small discs (1/4 in. diameter) of No. 3 filter paper were immersed in these solutions for one minute, then they were drained and plated at 2-in. intervals in rows on a large sheet of inoculated agar. After incubation for two days at room temperature, the culture indicated a difference between the different fungicides in toxicity to the test organism and in the way they were absorbed by the filter paper discs from the original solutions or the way they diffused from the discs into the agar. The amount of fungicides E and F that diffused from the discs appeared to be related to the concentration, in the range used, of fungicide solution in which the discs had been immersed. On the other hand, the amount that diffused in the case of fungicides A, B, and H appeared to be about the same for all concentrations. Fungicide G was not toxic to the inoculum on the agar sheet even at the highest concentration tested. The appearance of an agar sheet, on which discs of filter paper soaked in solutions of fungicides A, E, G, and F were placed, is shown in Fig. 3.

Conclusions

It seems evident that the plating technique described above is simple to follow, that it does not require expensive apparatus, and that it does not require the time and labor usually prerequisite to many other plating techniques. Its other advantages seem to be that the duration of a test is short, that the large area available for testing on each agar sheet allows the simultaneous testing of many different items, and that the data obtained may be readily subjected to statistical analysis. Other advantages may be discovered when this technique comes into wider use.

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NITROGEN DETERMINATION IN WHEAT LEAVES[1]

By D. W. A. Roberts[2]

Abstract

A survey of the more promising modifications of the Kjeldahl method for nitrogen determination indicates that the method using mercuric oxide as the catalyst as recommended by the Association of Official Agricultural Chemists is the most satisfactory. However small discrepancies were found when this method was applied to wheat leaves fractionated into soluble and protein (actually coagulable and indiffusible) nitrogen fractions. This method includes all the nitrate nitrogen in wheat leaves together with the organic nitrogen even when no pretreatment with salicylic acid is used. A simple and satisfactory method of separating coagulable and indiffusible nitrogen from soluble nitrogen in wheat leaves and seedlings is described.

Introduction

In connection with the physiological studies of the first leaf of Khapli Emmer wheat, *Triticum dicoccum* Schubler var. Khapli, it became desirable to investigate the distribution of coagulable and indiffusible nitrogen (mainly protein and hereafter referred to for brevity as protein nitrogen) and of soluble, diffusible nitrogen in the different segments of the leaf. Since earlier work in this laboratory on whole leaves had revealed certain discrepancies in the methods used for these determinations, the methods have been reinvestigated and improved in their application to the leaves and extended by a few data on their application to the grains and germlings of Khapli wheat. The results are presented in the belief that they will be of value to other workers engaged in the analysis of similar botanical materials.

Preliminary experiments on the leaves revealed small discrepancies which could only be accounted for by assuming that the Kjeldahl method used was not giving accurate results. Consequently a survey of the more promising modifications of the Kjeldahl method was undertaken in an attempt to obtain better results. This survey was followed up by an investigation of the fractionation process.

It is well known that, unless special precautions are taken, the Kjeldahl method does not give quantitative results for nitrogen in compounds not having the nitrogen directly connected to both carbon and hydrogen, or for ring nitrogen compounds. On the other hand the Dumas method of nitrogen determination has been found satisfactory for all types of organic nitrogen compounds. The latter method requires, however, that the substance to be analyzed must be reduced to a powder which can be mixed quantitatively with copper oxide. This can not be accomplished with the gummy residues

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of the water soluble nitrogen fraction of the leaves. Consequently the Kjeldahl method has been used on the leaves and in subsequent experiments on the grains and germlings.

The Kjeldahl Method

A survey of the recent literature reveals that four catalysts viz. potassium sulphate, copper, selenium, and mercury are frequently used in the Kjeldahl method. The more promising of these have been tested. Although A.O.A.C. (1) recommends two hours' digestion time as sufficient, Chibnall (4) claims that eight hours or longer are needed for some plant materials, and Shirley and Becker (10) have found three or four hours' digestion necessary for ring nitrogen compounds, even when mercury is used as a catalyst. Nitrates also interfere with the unmodified Kjeldahl method. Whitehead and Olson (12) have found that nitrate concentration is sufficiently high in some plant materials to produce interference. Since the first leaves of Khapli wheat contain considerable nitrate, this point has been investigated. The only other modification of the Kjeldahl method which has received much recent attention is the use of sodium hydrosulphite to determine azo and nitro nitrogen. This method was described by Simek (11). A modification of this method was tested in the hope that it might eliminate the small discrepancies which have been observed.

Comparison of Catalysts

Of the four catalysts mentioned previously, selenium seems to be the least satisfactory. It is not mentioned in A.O.A.C. (1) and has been found unsatisfactory for some types of biological materials by Osborn and Krasnitz (7), and unsuitable for aromatic amines and heterocyclic nitrogen by Korek (6). In view of these findings and the fact that heterocyclic nitrogen (as in chlorophyll and catalase) occurs in the leaves, selenium was not tested as a catalyst. Copper, mercury, and potassium sulphate were tested as catalysts on fresh greenhouse grown wheat leaves of known age.

In this set of experiments the salicylic acid modification was used in all cases. Potassium sulphate and mercury were tested by following the appropriate instructions given in A.O.A.C. (1). In testing copper as a catalyst the Gunning method was used except that 5 gm. of potassium sulphate and 0.5 gm. of copper sulphate were added to the digestion in place of the usual 10 gm. of potassium sulphate. In all these experiments six hours' digestion was used. Under these conditions it was found that there was serious danger of losing all the nitrogen when the Gunning method was used. According to Bradstreet (2), this occurs when the composition of the mixture approaches that of the acid sulphate. This is visibly indicated by the solidification of the digest on cooling.

Table I shows that there is not much difference between the three catalysts when a long period of digestion is used. However, the mercury catalyst method appears to give a slightly larger result. When mercury is used, the

TABLE I

TOTAL NITROGEN AS PERCENTAGE FRESH WEIGHT IN LEAVES ANALYZED USING THREE DIFFERENT CATALYSTS

Age of leaves, days	Potassium sulphate	Copper sulphate	Mercuric oxide
18	0.521	0.530	0.564
18	0.535	0.532	0.519
14	0.761	—	0.716
14	0.734	—	0.830
Average values for comparison	0.638	—	0.657

clearing is faster than when either potassium sulphate, or copper and potassium sulphate are used, and there is less danger of loss of nitrogen than when the modified Gunning method is used. Consequently the use of mercury was adopted.

Duration of Digestion

Table II gives the results of analyzing 19-day-old leaf samples and seeds by the adopted mercury catalyst method using different digestion times.

TABLE II

EFFECT OF DIGESTION TIME ON TOTAL NITROGEN DETERMINATIONS

Digestion time, hr.	Leaf nitrogen, % fresh weight	Seedling nitrogen, mgm. per seedling
1	—	1.41
2	0.62	1.36
4	0.63	1.41
6	0.61	1.43
8	0.62	—

The data show that a digestion period of three hours with leaves and two hours with seeds gives wide margins of safety. The long digestion times recommended by Chibnall (4) are not necessary for wheat when mercury is used as the catalyst.

Nitrate Interference

As a preliminary to investigating the nitrate interference in the whole leaves, a set of experiments was carried out using pure substances. The analytical methods used were the same as those described above, except that the preliminary treatment with salicylic acid and sodium thiosulphate was omitted.

Mixtures of known amounts of asparagin, potassium nitrate, and glucose were analyzed. Table III shows that the recovery of nitrate nitrogen is greater when the ratio of sugar to nitrate is greater. The mercury catalyst

TABLE III

NITRATE INTERFERENCE IN THE KJELDAHL METHOD WITH PURE SUBSTANCES

Catalyst used	Wt. of glucose, gm.	Wt. of potassium nitrate, gm.	Wt. of asparagin, gm.	Wt. of excess nitrogen recovered, mgm.	Nitrate nitrogen recovered, %
Potassium sulphate	0.1579	0.0299	0.2654	0.17	4.2
	0.2027	0.1686	0.0885	0.40	1.7
Copper sulphate	0.1577	0.0296	0.2572	2.98	74.6
	0.2027	0.1679	0.0768	9.32	39.6
Mercuric oxide	0.1580	0.0296	0.2677	3.72	92.3
	0.2031	0.1315	0.0905	9.53	52.4

gives the greatest recovery of nitrate nitrogen, the copper catalyst a large recovery, while the Gunning method gives a very low recovery of nitrate nitrogen. Of the three methods used, it will be noted that the procedure giving the fastest clearing gave the greatest nitrate nitrogen recovery. These results are in agreement with those of Whitehead and Olson (12).

In view of the fact that nitrate interference varies in magnitude with the proportion of carbohydrate present, it seemed possible that the discrepancies encountered in previous experiments with wheat leaves divided into protein and soluble nitrogen fractions might be due to nitrate interference. To settle this point two sets of experiments were carried out on fractionated and whole leaves. The method of fractionation will be described later. Analyses were made on similar samples of leaves using copper as a catalyst together with pretreatment with salicylic acid in one case but not in the other. Only three hours' digestion was used. The data in Table IV show that nitrate interference

TABLE IV

TEST FOR NITRATE INTERFERENCE USING COPPER SULPHATE AS A CATALYST. THE PERCENTAGE NITROGEN IN LEAVES CALCULATED ON A FRESH WEIGHT BASIS

Method	Green leaves	Fractionated leaves			
		Soluble and protein combined	Soluble alone	Protein alone	Sum of soluble and protein
No salicylic acid	0.587	0.578	0.213	0.384	0.597
With salicylic acid	0.537	0.553	0.222	0.346	0.568

is not a serious matter. However the nitrogen recovery with added salicylic acid is consistently lower than is the case when it is omitted. The reason for this has not been investigated but a test was made on whole leaves using mercury as a catalyst and three to four hours' digestion to discover whether a similar discrepancy occurred or not. In this case no discrepancy was found and it was also seen that the unmodified mercury catalyst method included all the nitrate nitrogen.

The Hydrosulphite Modification

The original method for determining azo and nitro nitrogen as described by Simek (11) had to be modified slightly for use with the seeds and leaves of wheat since these can not be dissolved completely before digestion. In the present tests the sample (20 seeds or *ca.* 15 gm. of green leaves) was refluxed for one hour with 5 gm. of sodium hydrosulphite dissolved in water (20 ml. for the seed samples and 60 ml. for the leaf samples). The solution was then evaporated to dryness under reduced pressure at 40° C. After decomposing the sodium hydrosulphite with 10 ml. of concentrated sulphuric acid, the remainder of the sulphuric acid containing the salicylic acid was added and the determination completed using mercury as a catalyst. Duplicate samples of seeds and leaves with and without the hydrosulphite modification were analyzed. The results showed that the same amount of nitrogen is recovered regardless of whether or not the hydrosulphite modification is used.

Fractionation into Soluble and Protein Nitrogen

Yemm (13), who investigated the different methods of separating protein and soluble nitrogen fractions in barley leaves, found very little difference between using boiling water and ether water for extracting the soluble nitrogen fraction. He also found very little difference in the values obtained for protein nitrogen regardless of whether he precipitated the protein with boiling water, colloidal iron, or trichloroacetic acid. In view of these findings Brodie (3) used boiling water to extract the soluble nitrogen, and to coagulate the protein nitrogen. Since this method is very simple and convenient it was employed.

Method of Fractionation

A 50 leaf (*ca.* 5 gm.) sample was cut into small pieces and placed in a Kjeldahl flask to which was added 150 ml. of boiling water. The leaves were boiled under a reflux for 25 min. and allowed to cool for nearly an hour. The leaf residue was then separated from the liquid by straining the mixture through a Gooch crucible without asbestos. The flask and leaf residue were then washed with approximately 150 ml. of boiling water. The leaf residue was subjected to the Kjeldahl determination and the result referred to as the protein nitrogen. The soluble fraction was then evaporated to dryness under reduced pressure at 40° C. and its nitrogen content determined and called soluble nitrogen.

For determinations of protein and soluble nitrogen in the grains and germlings a similar method was used. Forty grains or seedlings were found to make a suitable sample and these were not cut up or ground up before being added to the Kjeldahl flask. All the other details were the same as in the analysis of the leaves.

Duration of Extraction

In choosing the correct extraction time, care must be taken that the extraction is continued long enough to remove all the soluble nitrogenous compounds but not so long that the acidic water extract causes hydrolysis of the proteins and subsequent leaching of the products of hydrolysis into the soluble nitrogen fraction. Table V gives the results of a series of experiments in which comparable samples of leaves and one-day-old seedlings were boiled for different

TABLE V

THE EFFECT OF THE DURATION OF EXTRACTION ON THE PROTEIN AND SOLUBLE NITROGEN FRACTIONS IN WHEAT LEAVES AND SEEDLINGS

Time of boiling, min.	Leaves				Seedlings	
	Nitrogen, % fresh weight				Nitrogen, mgm. per plant	
	Expt. 1		Expt. 2		Expt. 3	
	Soluble	Protein	Soluble	Protein	Soluble	Protein
10	0.276	0.476	0.172	0.373	—	—
20	0.282	0.470	0.185	0.381	0.08	1.30
30	0.263	0.459	—	—	0.07	1.21
60	—	—	0.189	0.381	0.09	1.22
240	—	—	0.242	0.347	—	—

NOTE: *Average value for total nitrogen for eight comparable samples of leaves, 0.651.*
Average value for total nitrogen for four unfractionated samples of leaves, 0.642.

periods of time to separate the protein and soluble nitrogen fractions. No detectable protein hydrolysis was found in the first hour of boiling but protein degradation is quite evident after four hours of boiling. Extraction was found to be complete in 10 min. Twenty-five minutes was adopted as the boiling time since this ensures complete extraction without any danger of protein hydrolysis.

The Use of Asbestos for Filtering

The use of asbestos in the Gooch crucible for separating the protein and soluble nitrogen fractions was found to be unnecessary and undesirable. With the washing that was used (150–200 ml. boiling water) the asbestos retains nitrogen corresponding to 0.002–0.004% of the fresh weight of the leaves analyzed. This amounts to about 1% of the total nitrogen in the leaves.

Acidulation of the Extracts

Acidulation of extracts for nitrogen determinations is used to avoid loss of ammonia when the extracts are evaporated to dryness. Experiments were made to test this in the case of wheat leaves and also to investigate the possibility of losing nitrate nitrogen by excessive acidulation.

Extracts containing the soluble nitrogen were made in large volumes by the method described above. For these experiments 290 gm. of 27-day-old wheat plant tops were boiled in five liters of distilled water for 20 min., allowed to cool, and then filtered. Three hundred ml. aliquots of the filtrate at pH 5.9 were adjusted to the desired pH with dilute hydrochloric acid, dilute sulphuric acid, or dilute sodium hydroxide, and then evaporated to dryness under reduced pressure at 40° C., and analyzed for nitrogen by the method adopted above. In the experiments on acidulation the nitrogen which was lost during evaporation to dryness was trapped by drawing all the vapors from the soluble nitrogen liquid through 300 ml. of 0.1 *N* sodium hydroxide. In these experiments both solutions evaporated simultaneously. The trapped nitrogen was determined in the usual way. In order to compare the quantity of trapped nitrogen with the nitrate nitrogen, the latter was determined by the method of Robertson described by Prince (8, 9).

The data in Table VI show that no important loss of nitrogen occurs when the evaporation is carried out between pH 2.0 and pH 9.2. The amount of trapped nitrogen increases as the acidity of the soluble nitrogen extract is

TABLE VI

THE EFFECT OF pH DURING EVAPORATION ON THE RECOVERY OF SOLUBLE NITROGEN FROM WHEAT LEAVES

pH	Soluble nitrogen, mgm. per 300 ml.	Trapped nitrogen, mgm. per 300 ml.	Total nitrogen, mgm. per 300 ml.
2.0	31.0	0.8	31.8
3.0	31.6	0.4	32.0
5.9	30.5	0.1	30.6
7.0			31.4
9.2			31.4

NOTE: *Nitrate nitrogen in 14-day-old tops 11.0% of total soluble nitrogen.*
Nitrate nitrogen in 27-day-old tops 9.0% of total soluble nitrogen.

increased. Excessive acidulation must therefore be avoided. At pH 2.0 the loss of soluble nitrogen during evaporation is much less than the nitrate nitrogen present.

Discrepancies in the Adopted Procedure

Green leaves, fractionated leaves, and fractionated leaves with the two fractions combined were analyzed using mercuric oxide as the catalyst. The results are given in Table VII (compare also Table V) which shows a slight

TABLE VII

TOTAL NITROGEN AS PERCENTAGE FRESH WEIGHT IN FRACTIONATED AND UNFRACTIONATED WHEAT LEAVES, MERCURIC OXIDE USED AS A CATALYST IN DETERMINATIONS

Age of leaves, days	Not fractionated	Fractionated leaves			
		Two fractions combined	A-Soluble nitrogen	B-Protein nitrogen	Sum of A and B
15	0.707	0.736	0.270	0.445	0.715
17	0.546	0.595	0.252	0.342	0.594

increase in nitrogen when the leaves are fractionated. From the data presented here it is doubtful whether the increase in nitrogen due to fractionating is significant; but a statistical analysis of all the experimental data (27 cases), regardless of the Kjeldahl modification used, does indicate that a significant increase in total nitrogen is a result of fractionating the nitrogen. This increase appears to be of the order of 4% of the total nitrogen. In treating the data, the percentage increase in nitrogen resulting from fractionation in sister samples was determined. Using Student's t method (5) the mean value of the percentage increase in nitrogen after fractionation was found to be significantly greater than the expected value of zero per cent. It is thus apparent that the adopted procedure is not entirely satisfactory for the determination of wheat leaf nitrogen. The discrepancies are small and are apparently influenced by the pH at which the soluble nitrogen fraction is evaporated (cf. Table VI).

In the case of seedlings an insufficient number of experiments has been performed to detect discrepancies as small as those shown to occur in the leaf determinations. There is no great change in the total nitrogen of seedlings as a result of fractionation, nor is there any appreciable change in the total nitrogen content of germinating Khapli Emmer wheat seedlings during the first five days of growth at 22° C. when supplied with distilled water only. The method adopted is therefore satisfactory for nitrogen determinations in the young seedlings.

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QUALITATIVE AND QUANTITATIVE DETERMINATION OF THE WHEAT LEAF CARBOHYDRATES[1]

By D. W. A. Roberts[2]

Abstract

The nonreducing sugar in wheat leaves is probably entirely sucrose. It is the only abundant sugar. Free reducing sugars are absent, or almost absent from wheat leaves grown under the conditions described. The reducing power in the cleared alcoholic extracts of the leaves is, at least, partly due to degradation products of ascorbic acid. Other nonsugar reducing substances also are apparently present. The alcohol insoluble residue from wheat leaves contains little or no fructosan, dextrin, or starch at the two and one-half week old stage. Satisfactory methods for extracting and determining the sucrose are described. The following methods gave satisfactory results with wheat leaf extracts: the reducing power methods of Hanes and Somogyi after acid or preferably invertase hydrolysis, Ost's solution for fructose residues, the method of Neuberg and Strauss, the colorimetric resorcinol method. The latter method gives only approximate values for fructose residues unless the sugar concentration is high; the method is then fairly reliable. The following methods did not give satisfactory results: Hanes and Somogyi methods for free reducing sugars initially present in the extract, the hypoiodite titration for aldose sugars, and Sieben's method for fructose determination.

Introduction

An examination of the carbohydrate distribution in the leaves of *Triticum dicoccum* Schubler var. Khapli was started in an attempt to shed some light on the metabolic basis of the respiratory gradient found in the leaves. In view of the fact that no universally acceptable method of sugar analysis is available for the analysis of plant extracts, some time was spent in examining some of the methods available. As a necessary preliminary step, an investigation into the nature of the carbohydrates and some of the interfering substances present in the leaves was undertaken.

A review of the literature reveals that there is uncertainty as to the identity of the carbohydrates in the leaves of wheat. Belval (6) claims that glucose, fructose, and sucrose are the sugars present in wheat leaves. This conclusion is based on studies using a polarimeter and invertase. He found no trace of fructosans in wheat leaf blades, but did find them in the sheaths. Newton (28) claimed that sucrose was the only nonreducing sugar present because he found that hydrolysis with hydrochloric acid gave the same value for the quantity of nonreducing sugar as the citric acid hydrolysis of Davis and Daish (11). Like Belval, he found no starch in wheat leaves. De Cugnac (12), who studied the carbohydrates of many grasses, concluded that the wheat leaf blades contained glucose, fructose, and sucrose only. Yemm (47) who studied the carbohydrates of barley leaves, concluded that glucose, fructose, sucrose,

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fructosans, and sometimes starch were present in the leaves. He relied on the results of reducing power determinations, after various treatments, to decide what the carbohydrates were. Fructosans have since been isolated from barley leaves by Archbold and Barter (5), and from *Poa trivalis* by Challinor, Haworth, and Hirst (9). The latter authors found nearly equal quantities of free glucose and fructose but no sucrose in the leaves. However they used water extracts of dried grass and so the sucrose, if present originally, may have been hydrolyzed. Archbold (4) later concluded that fructosans were present in large quantities in barley leaves only under two conditions viz. (*a*) when the percentage of nitrogen was low and the percentage of carbohydrates high, and (*b*) when the plants were at least 10 weeks old. Except in the case of sucrose none of these sugars ever seem to have been isolated from wheat leaves. Phillis and Mason (30), who studied the carbohydrates of cotton leaves, reviewed the literature on the presence of sucrose in leaves, and were not satisfied that its presence had been conclusively demonstrated in leaves. Isolations of sucrose have been made by Schulze and Frankfurt (38) and Schulze (37) from the tops of rye, oats, and *Lolium italicum*. In all three cases, these authors found fructosans also present. These authors obtained sucrose from the leaves of *Alnus* sp. but failed in other cases. Phillis and Mason (30) were unable to demonstrate the presence of free glucose and/or fructose by using the phenylhydrazine reaction in cotton leaves which had been darkened for some hours previous to harvesting. In the case of illuminated leaves, they do not state how long they found it necessary to heat their extracts with phenylhydrazine and sodium acetate before they obtained any osazone. In mixtures suspected of containing sucrose, glucosazone must precipitate within the first 20 min. of boiling, otherwise it is not safe to conclude that any free reducing sugar was present originally. Since Phillis and Mason presented evidence which makes it reasonably certain that sucrose is present in cotton plant leaves, there is some room for doubt as to the presence of free fructose and free glucose in those leaves. A survey of Chemical Abstracts reveals only two cases in which sucrose has been isolated from leaf material since the paper by Phillis and Mason. Fondovila and Diaz (15) have isolated sucrose from yerba maté (dried leaves of *Ilex paraguayensis* St. Hil.). Putman, Hassid, Krotkov, and Barker (31) have isolated sucrose from the leaves of *Canna indica* and present good evidence for its presence in tobacco leaves. These authors state that glucose and fructose are present in *Canna* leaves but they do not present evidence to prove this point. In all cases their isolations of these sugars were made after the hydrolysis of starch or sucrose.

Reducing substances are found in the alcohol extracts of most plant materials. Authors who have been interested in carrying out sugar determinations have usually assumed that those reducing substances remaining in the extracts after treatment with neutral or basic lead acetate were reducing sugars. This assumption is based on the formation of osazones with phenylhydrazine, a reaction which must be carried out with due regard to interference from sucrose, when present, if the evidence is to be conclusive of the

presence of free reducing sugar. However Szent-Gyorgyi (42) found that cabbage leaves and orange juice contained ascorbic acid, a powerful reducing substance. This substance has also been isolated from lemon juice (44) and paprika (41). If the very rapid decolorization of 2,6-dichlorophenolindophenol in acid solutions is a specific test for the presence of ascorbic acid, then this substance must be almost universally distributed in plant materials in appreciable quantities. Under these circumstances it is a serious potential source of interference in reducing sugar determinations. The interference from this source will be considered in the case of wheat leaves.

Qualitative Tests for Sugars

For these preliminary investigations, greenhouse and outdoor grown Khapli Emmer wheat plants were used. The whole tops of the plants two to three weeks old were used. The tops were extracted with boiling 80% alcohol. The alcoholic extract was evaporated to dryness at 40° C. under reduced pressure and the solids subsequently taken up in distilled water. This aqueous solution was cleared with saturated neutral lead acetate solution and the lead was removed by the addition of solid sodium carbonate. The aqueous solution thus obtained was subjected to the color tests described below. In carrying out these tests the methods described by Browne and Zerban (7) were used.

A positive Seliwanoff's test was obtained. This indicated the presence of ketohexose residues either as the free sugar or in the combined state.

With the phloroglucinol test a red solution was obtained which showed an absorption band in the yellow when examined with a spectroscope. With the orcinol reaction a dark colored solution was obtained, the amyl alcohol extract of which showed an absorption band in the yellow. Both these tests indicate the presence of pentose or hexuronic acid residues. In some of the extracts negative tests have been obtained with these two reactions after the extracts were cleared with basic lead acetate. The significance of these results will be considered later.

The Raybin test was weak but positive on extracts cleared with basic lead acetate. This test does not work satisfactorily with dilute sucrose solutions. The only sugars known at the present time to give a positive Raybin test are those which contain glucose linked to a ketohexose or ketopentose by a linkage strictly comparable to that in sucrose. These sugars are sucrose (33), raffinose (33), gentianose (34), stachyose (34), α-*d*-glucosido-β-*d*-sorboside (13), and α-*d*-glucosido-β-*d*-ketoxyloside (13).

Considerable difficulty was experienced in the first few attempts to prepare an osazone from wheat leaf extracts. In view of subsequent findings, it is probable that this difficulty arose chiefly from the very low quantities of sugar residues present in the extracts which were used. High sugar leaf extracts yield abundant osazone. For this test 200 gm. of greenhouse grown wheat leaves harvested in the late afternoon were ground in a "Health Mine" juicer

and the juice run into saturated lead acetate solution to inactivate the invertase and precipitate some of the nonsugars. The precipitate was centrifuged down and discarded. Saturated sodium bisulphite solution was used to remove the excess lead. The use of this deleading reagent has been found to give better results than carbonate if a subsequent test with phenylhydrazine is to be made. After neutralization with 25% potassium hydroxide the solution was treated with phenylhydrazine hydrochloride and sodium acetate in a boiling water bath. Nothing precipitated from the hot solution within the first 20 min. in the boiling water bath. The solution was then cooled when a few osazone-like crystals separated out with a lot of amorphous matter. This was filtered off but was too small in quantity to examine further. The filtrate was heated in the boiling water bath further and eventually a large yield of crystals microscopically resembling glucosazone were obtained. The final yield was too large to be accounted for as coming only from the reducing substances as determined by the Hanes method (17). The long delay period which is always encountered in preparing osazone from wheat leaf preparations lends support to the view that the yield comes mostly, if not entirely, from some easily hydrolyzable nonreducing sugar such as sucrose. It is possible that wheat leaf extracts contain substances which delay osazone formation from free reducing sugars. When the osazone obtained was recrystallized twice from 50% alcohol, it was found to have an unchanged corrected melting point of 208°–209° C. on rapid heating. This agrees with values of 208°–210° C. quoted in the literature for glucosazone. On these grounds it is concluded that the only sugar residues existing in the free form, or as easily hydrolyzable nonreducing sugars in wheat leaves, are glucose and fructose. Mannose is ruled out since no indication of the presence of its insoluble phenylhydrazone has been found in any of the osazone preparations made.

An attempt to prepare potassium gluconate from an alcoholic wheat leaf extract cleared with neutral lead acetate and sodium carbonate was not successful. The procedure of Moore and Link (25) was followed in principle, except that a 4% methanolic potassium hydroxide solution was added to the methanolic solution of the wheat leaf solids before treatment with iodine, in an attempt to precipitate substances which would otherwise be precipitated with the potassium gluconate. This precipitate was found to contain the wheat leaf nonreducing sugar. Subsequent experiments with hydrogen sulphide show that sodium carbonate does not remove all the lead from wheat leaf extracts. It seems likely that the residual lead in the preparation was responsible for the precipitation of the nonreducing sugar at this stage of the preparation. Sucrose is not precipitated from methanolic solution by the addition of methanolic potassium hydroxide.

The nonreducing sugar found in the potassium hydroxide precipitate was further purified by precipitating it from an aqueous solution by neutral lead acetate and ammonium hydroxide. The precipitate was decomposed by 25% sulphuric acid, freed from sulphate with barium acetate, and the supernatant

evaporated to dryness under reduced pressure. The solids were dissolved in methanol and precipitated with five volumes of acetone. The precipitate was stored in a desiccator.

This crude wheat leaf sugar preparation assayed 25% nonreducing sugar, which was completely hydrolyzed by invertase, and later was shown to contain approximately 50% fructose residues by means of the colorimetric Seliwanoff method to be described later. The preparation was hydrolyzed in the presence of 3 *N* hydrochloric acid at 55° C. Table I shows that the progress of hydrolysis is similar in sucrose and wheat leaf nonreducing sugar.

TABLE I

COMPARISON OF THE HYDROLYSIS OF WHEAT LEAF NONREDUCING SUGAR WITH SUCROSE

Duration of hydrolysis, hr.	Resulting reducing power by Hanes method, ml. of 0.01 *N* thiosulphate	
	Wheat leaf sugar	Sucrose
0.25	1.05	1.08
0.50	1.07	1.17
1.0	1.09	1.05
2.0	1.05	1.00
3.0	1.03	0.98
4.0	1.01	0.97
7.0	0.98	0.98
24.0	0.98	0.96

Quantitative Methods for Sugar Determination

A survey has been made of some of the quantitative methods available for sugar determinations on the wheat leaf extracts. Three objectives were kept in mind during this survey. The first was to attempt to find out which methods give reliable results as measured by the agreement between methods relying on different chemical reactions. The second was to establish the proportion of glucose and fructose present in hydrolyzed and unhydrolyzed extracts. Finally, it was hoped that satisfactory methods could be found for use in the routine analyses to be undertaken in connection with the studies on the metabolic gradients in the wheat leaf.

The Hanes modification of the Hagedorn and Jensen method was tested (17). According to Wood (46), the amount of ferricyanide reduced depends upon the pH at which the reduction occurs. This point was tested in the case of a mixture of equal weights of glucose and fructose and wheat leaf extract. In these experiments the 5 ml. volume to be analyzed was made up of 3 ml. of sugar solution, an appropriate amount of 5% acetic acid solution, and sufficient water to make the volume up to 5 ml. By varying the quantity of acetic acid solution used, different pH values were obtained. The pH was determined in parallel experiments after the addition of the ferricyanide reagent. The pH values given in Table II are thus the values at which the reduction actually

TABLE II

EFFECT OF pH UPON THE REDUCING POWER OF SUGAR AND WHEAT LEAF EXTRACT AS DETERMINED BY THE HANES METHOD

pH	Ml. of 0.01 *N* thiosulphate used			
	Sugar	Wheat leaf extract	Mixture	Calculated for mixture
4.7	0.00	0.56	0.60	0.38
8.8	4.32	1.84	5.06	4.10
10.8	5.32	1.87	7.66	4.71

began. The data show that discordant results were obtained between the sugar solution, the wheat leaf extract, and a mixture of the two. The mixture used was made up of equal volumes of wheat leaf extract and sugar solution. Comparing the wheat leaf extract with the sugar solution it will be noted that the wheat leaf extract reduces acid ferricyanide whereas sugar solution does not. Further there is little difference in the reducing power of wheat leaf extract between pH 10.7 and pH 8.8 whereas there is a considerable difference between the reducing powers of sugar at these two pH values. It is apparent also that the reducing powers of the mixture can not be calculated from the reducing powers of the two solutions separately. This suggests that something in the wheat leaf extracts alters the reducing power of the added sugar. The data also show that a part, or possibly all of the reducing power (if we consider the reducing powers of the sugar solution and the extract at pH 10.7 and pH 8.8) in the wheat leaf extract, is due to nonsugars.

The original copper micromethod of Somogyi (40) was tested since it was the only copper micromethod recommended in A.O.A.C. (29) for any purpose. This method has been criticized (39) because iodide is present in the solution in which reduction occurs. The iodide is said to hold reduced copper in solution so that it can be reoxidized by molecular oxygen. Thus the method is supposed to give low values. However it has also been claimed that the presence of iodides during the reduction reduces the sensitivity of the copper reagent to nonsugar reducing substances (39). It will be seen later that this reagent is much less sensitive to the degradation products of ascorbic acid than is the Hanes method. Fairly good agreement was found in a set of routine experiments between this method and the Hanes method in all cases where sugar was known to be present in appreciable quantities. In the reducing sugar determinations this method gave consistently lower results than the Hanes method but there is no reason to think that the true quantity of reducing sugar present, if any is present at all, is too low to determine by any of the methods at present available.

Several attempts were made to use the hypoiodite titration following, in general principles, the method of Kline and Acree (23) but although satisfactory results were obtained with hydrolyzed sucrose, only discordant results

were obtained with wheat leaf extracts. The extracts were found to absorb a great deal more iodine than was equivalent to the alkali used up.

The use of Ost's solution for the determination of fructose was found to be satisfactory as long as the fructose concentration in the extract was not very low. Unfortunately rather large samples are necessary to employ this method and, consequently, it was impracticable to use this method for the routine analyses of sugars required in the study of the gradients in the wheat leaf. Ost's solution was made up and the reduction carried out in accordance with the directions given in Browne and Zerban (7). The precipitated copper was removed by filtration, washed thoroughly, and determined by the method of Hill (20). This method was used in preference to that recommended by Jackson and Mathews (21) since the final titration has a much more readily determined end point. The tables given by Jackson and Mathews were used to convert the precipitated copper into its equivalent of fructose.

The method of Neuberg and Strauss (27) employing 2,4-dinitrophenylhydrazine was tested and found to be satisfactory for use when large quantities of sugar were present in the samples available for analysis. In the experiments with this method reported later, the samples were evaporated to dryness prior to the determination in order to facilitate the adjustment of the final sugar concentration to a value in excess of 0.2% as specified by the authors. The reaction was carried out in a Kjeldahl flask with its mouth closed by a sealed glass bulb instead of a reflux condenser. In other respects, the recommendations of the authors were followed. The quantity of total sugar residues was then calculated on the assumption that the precipitate was the 2,4-dinitrophenylosazone of glucose. This method included the sugar phosphates, pentoses and their derivatives, glucuronides, aldehydes, ketones, ascorbic acid, dehydroascorbic acid, and possibly other ascorbic acid degradation products unless these substances were previously removed by the clearing reagents used.

Following the directions given in Wiechmann's book on sugar analysis (45), Sieben's method for the destruction of fructose by means of acid was tested. The determination of the quantity of fructose destroyed was found to depend on whether the determinations of residual sugar were made by Hanes's method or the method of Somogyi. Sieben's method was therefore considered to be unsatisfactory. However in both cases the reducing power fell from the start of the experiment, and thus failed to give any evidence for the presence of any nonreducing sugar which was difficult to hydrolyze.

The colorimetric Seliwanoff method of Roe (36) was tested. Preliminary tests on a sugar rich wheat leaf preparation were very promising and so the method was modified for routine use with the Coleman Model 14 Junior Spectrophotometer. When, however, wheat leaf preparations containing little or no sugar were analyzed during the routine tests, the presence of other substances which develop color when heated with resorcinol and hydrochloric acid was revealed. An approximate correction for these substances was applied, as described below. Using this correction fairly good results were obtained with this method. It is a poor substitute for the use of Ost's

solution if sufficient material is available to make the use of the latter method possible. The resorcinol method is quick and convenient where its use is practicable.

Fig. 1 shows the absorption spectra of each of the following substances after treatment with resorcinol and hydrochloric acid: (1) fructose, (2) sugar rich wheat leaf extract previously cleared with lead acetate and sodium carbonate,

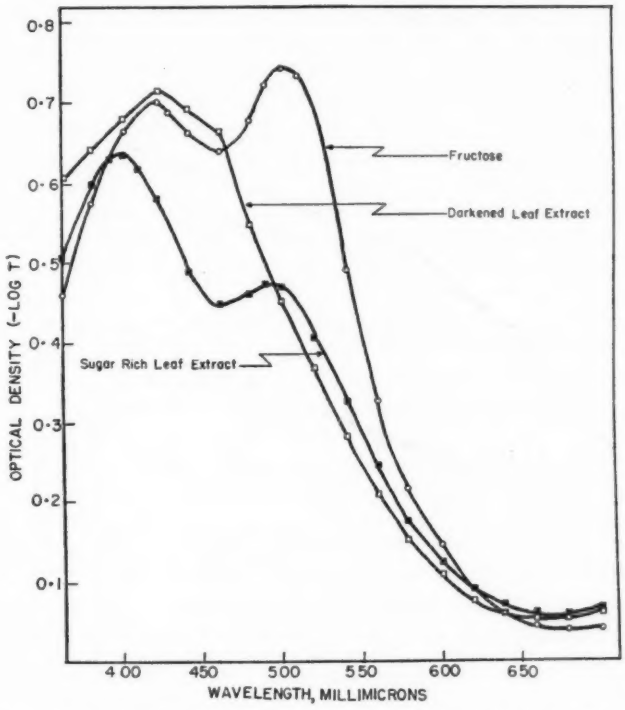

FIG. 1. *The absorption spectra of fructose, sugar rich wheat leaf extract, and extract from darkened wheat leaves after treatment with resorcinol and hydrochloric acid.*

and (3) extract from darkened leaves after a clearing treatment similar to that of case (2). This figure shows that a minor transmission maximum occurs at 460 mμ in both the fructose and sugar rich wheat leaf extracts and a minimum in the region of 490 to 500 mμ. The positions of the major absorption band in both sugar rich and starved wheat leaf preparations agree with each other but not with the position of the comparable band for fructose. Some of the possible interfering substances to blame for this discrepancy will be considered later. On the basis of experiments to be described shortly it is believed that the extracts from the darkened leaves contain little or no free, or

combined, fructose. Sucrose determinations on these leaves show practically none of that sugar present.

The use of standard fructose solutions is inconvenient and may be avoided when a suitable type of colorimeter is available provided that the method of treatment with resorcinol and hydrochloric acid is carefully standardized. One of the most important considerations in the standardization of the method is the duration of the heating time. This matter is stressed by Roe in the original paper. In the present set of experiments test tubes with a 5/8 in. bore were used. Fig. 2 shows the progress of the color development in a

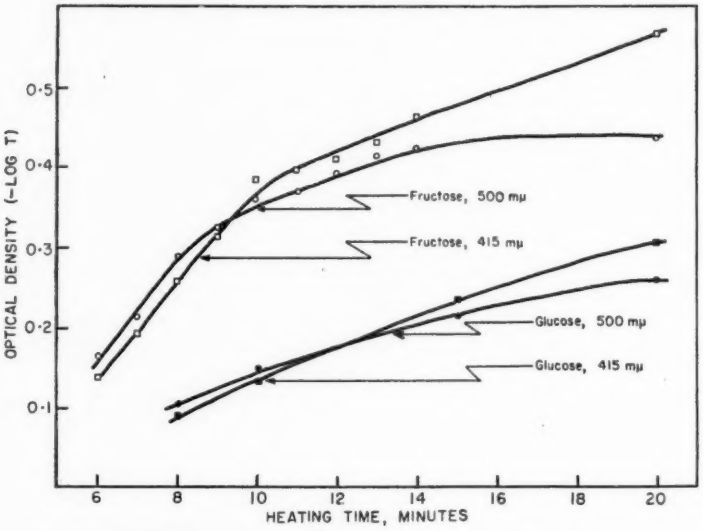

FIG. 2. *The optical density of fructose and glucose solutions at 500 mμ and 415 mμ after different heating periods at 80° C. with resorcinol and hydrochloric acid. The following solution concentrations were used, fructose 47 μgm. per ml., glucose 1000 μgm. per ml.*

fructose solution (concentration 47 μgm. per ml.) compared with that in a glucose solution (concentration 1000 μgm. per ml.). There are two considerations in choosing the best heating time to use. The first consideration is to choose a time so that small errors in the duration of the heating will not produce appreciable changes in the color developed. As long as it is possible to use only the absorption band at 500 mμ in the determinations this condition is satisfied by 15 min. heating at 80° C. The second consideration is the interference from glucose. As Fig. 2 shows the color developed by this interfering substance increases steadily from the start of the heating time. Consequently it is necessary, when glucose residues are present, to use the shortest heating time that is compatible with the first consideration mentioned above. As a compromise 15 min. was chosen in the present set of experiments. With the

test tubes used in these experiments the eight minute heating time recommended by Roe is very unsuitable for use in the absence of standard fructose solutions since it lies on the steepest part of the curve of color development plotted against time.

Using the reagents described in the paper by Roe (36) but increasing the heating time at 80° C. from 8 min. to 15 min., a calibration curve for the method was constructed, using solutions of known strengths of fructose and light with a wave length of 500 mμ. For concentrations of fructose below 80 μgm. per ml., the Lambert Beer Law was found to hold well. The data presented in the following section of this paper were determined from the single reading using light of 500 mμ wave length.

In the sugar determinations which will be reported in subsequent papers an approximate correction for the interfering substances was applied. This correction is based on a number of assumptions. The interference is assumed to be due to a single substance or a group of substances whose concentrations relative to each other do not change from extract to extract. The substance after treatment with resorcinol and hydrochloric acid is assumed to produce a colored product obeying the Lambert Beer Law at both the wave lengths chosen for the determinations. Fructose is also assumed to obey the same law at both the chosen wave lengths. The only doubtful one of the above assumptions is that the interference is due to a single substance.

In the experiments to be reported in later papers, absorption measurements were made at wave lengths of 500 mμ and 430 mμ. The absorption spectrum given in Fig. 1 for darkened wheat leaves was arbitrarily said to have been produced from a solution containing one unit of interfering substance per ml. Knowing the optical density of a solution at two wave lengths, and the absorption spectra of given concentrations of the two components of the system, then the concentration of either of the components may be calculated as long as it is safe to assume that the optical density of the mixed solution is the algebraic sum of the values that would have been obtained for each of the components provided that the other had been absent. This latter assumption was also made.

The sucrose present in the leaves was determined by hydrolyzing a 10 ml. aliquot of the cleared preparation with 10 ml. of hydrochloric acid of sp. gr. 1.1 for 11 min. in a water bath at 70° C. After being cooled and allowed to stand for 30 min., the hydrolyzate was neutralized with 20% sodium hydroxide with cooling and the volume made up to 50 ml. The total reducing power after hydrolysis was determined by the Hanes and Somogyi methods described above. The increase in reducing power resulting from acid hydrolysis was calculated as sucrose. The increase in reducing power after hydrolysis with invertase was also determined. For this hydrolysis, 5 ml. of the cleared extract were added to 5 ml. of 0.2 *M* acetate buffer at pH 4.5 and 1 ml. of 0.2% invertase solution (solid British Drug Houses preparation). Three hours of digestion at room temperature were found to be more than sufficient

to hydrolyze all the invertase hydrolyzable sugar in the preparations. The digest was then neutralized with 0.1 *N* sodium hydroxide solution and made to volume. Reducing power determinations were then made. Reducing power blanks were carried out on the enzyme preparation. Since a zero blank value was obtained with the method of Somogyi this method was used in the routine tests to be reported in later papers.

The Identity of the Wheat Leaf Sugars

Experimental

The methods described above were applied to wheat leaf extracts to obtain further information as to the identity of the carbohydrates present. The extract used was a part of the material obtained from leaves grown in the greenhouse in December and harvested in the late afternoon. The leaves were ground in a "Health Mine" juicer and the juice run into 4 liters of 95% alcohol. The fibrous residue was also transferred to the alcohol. One kilogram of leaves was used. A 1600 ml. aliquot of this extract was evaporated to dryness at 40° C. under reduced pressure, after filtering to remove the cellular debris. The solids so obtained were taken up in approximately 100 ml. of water and cleared with basic lead acetate solution (sp. gr. 1.25). The lead was removed by sodium carbonate and the pH adjusted to 7.0. This solution was made up to 250 ml. and is subsequently referred to as the original solution.

Ten ml. of the original solution were diluted to 50 ml. and the "reducing sugar", nonreducing sugar, and invertase hydrolyzable sugar in this solution were determined by both the Hanes method and the method of Somogyi.

Five ml. of the original solution were made to 50 ml. Aliquots of this solution were used to determine the fructose residues by the colorimetric resorcinol method.

For the determination of free fructose in the extract, 20 ml. of the original solution were analyzed using Ost's solution as described above. A 10 ml. sample of the original solution was hydrolyzed with hydrochloric acid as described above, neutralized, and added to 50 ml. of Ost's solution. The fructose residues were then determined in the usual way. A 15 ml. aliquot of the original solution was hydrolyzed with invertase and the fructose residues determined by Ost's solution.

Two 25 ml. samples of the original solution were evaporated to dryness, and later analyzed by the method of Neuberg and Strauss as described above. This method determines total sugar residues.

The Reducing Sugars

The results of the sugar determinations outlined above are presented in Table III. The striking feature about the reducing sugar determinations given in this table is the disagreement between the results obtained by the different methods. It is also noteworthy that the value obtained for total sugar residues as determined by the method of Neuberg and Strauss agrees

TABLE III

SUGARS IN 400 GM. SAMPLE OF GREENHOUSE GROWN WHEAT LEAVES AS DETERMINED BY DIFFERENT METHODS

Method used	Free reducing sugar, mgm.	Acid hydrolyzable sugar, mgm.	Invertase hydrolyzable sugar, mgm.
Hames	515	775	950*
Somogyi	260	838	925*
Ost**	17	417	383
Colorimetric resorcinol**	512	422	485
Neuberg & Strauss	Total sugar residues as hexose, 816 mgm.		

* *Calculated as sucrose using the same calibration curve as for acid hydrolysis.*
** *Total fructose residues after given treatment.*

with the sucrose determination by the other methods. This indicates the virtual absence of free reducing sugar. Furthermore both Ost's solution and the colorimetric resorcinol method give values for fructose residues which suggest that most of the "reducing sugar" is not fructose. It should be remembered that the fructose determinations by Roe's method, given in Table III, are not corrected for the interfering substances and, consequently, are higher than the true value. The free fructose determined by Ost's solution is also too high as it has not been corrected for the presence of sucrose. The most satisfactory explanation of these results is that free reducing sugar is either absent from, or present only in very small quantities in, the leaves of Khapli wheat.

This latter conclusion is supported by the behavior of the reducing substance toward ferricyanide at different pH values (cf. Table II) and partially also by its behavior when it is precipitated by ammonium sulphate. The failure to prepare potassium gluconate also suggests that free glucose is present in much lower concentrations than expected from the reducing power determinations.

One important consideration remains to be examined before the view can be accepted that the so-called reducing sugar in the extracts from Khapli wheat leaves is not really sugar. Glucosazone can be prepared from wheat leaf extracts under suitable conditions. The final yield is too great to be accounted for as coming only from the reducing sugar, even if we assume that the values given by the Hanes method are correct. No osazone can be made from the darkened leaves which contain little or no sucrose. This result is in agreement with that of Phillis and Mason (30) for cotton plant leaves. It is probable that all or most of the glucosazone obtained from wheat leaf extracts comes from the hydrolysis of sucrose which occurs during the prolonged heating periods necessary to produce the glucosazone.

No unquestionably positive evidence for the presence of free reducing sugars in wheat leaves has been obtained. On the other hand the results of the other

experiments reported above suggest strongly that little or even none of the reducing power of the extracts of wheat leaves is due to reducing sugar. When these results are taken in conjunction with the failure of Phillis and Mason to prepare glucosazone from darkened cotton leaves the question arises as to whether leaves contain free reducing sugar in appreciable quantity. As pointed out earlier the results of osazone production must be considered as inconclusive unless the osazone formation occurs in the first 15 to 20 min. of heating except when it can be shown that sucrose or other readily hydrolyzed sugars are absent from the extract. In order to prove that there is no free reducing sugar in wheat leaves it will be necessary to isolate the reducing substances and show that the nonsugars account for all the reducing power in the extracts.

The Nonreducing Sugars

The very large increase in reducing power obtained when leaf extracts are hydrolyzed by acid or invertase indicates the presence of a nonreducing sugar. When wheat leaf extracts are treated with phenylhydrazine they yield, after a prolonged heating period, abundant quantities of glucosazone. This means that the component sugar residues are glucose and/or fructose. Mannose is ruled out since its insoluble phenylhydrazone has not been encountered in these experiments. Table III shows that the nonreducing sugar consists of approximately 50% fructose. The remainder must be glucose. This view is supported by the fact that the methods of Hanes and Somogyi agree fairly well as to the quantity of sucrose present in the extracts when measured by the increase in reducing power after hydrolysis. The methods of Hanes and Somogyi also agree with the method of Neuberg and Strauss (cf. Table III). Good agreement between methods is not so probable if the sugar has been incorrectly identified. In addition to the above, the nonreducing sugar is completely hydrolyzed by invertase, gives a positive Raybin test, and has a hydrolysis–time curve similar to that of sucrose (cf. Table I). The data make it very probable that the only alcohol soluble carbohydrate present in appreciable quantities in wheat leaves is sucrose. To prove that the nonreducing sugar is sucrose it will be necessary to isolate that sugar from the leaves. This isolation has been achieved by Ward.*

Evidence for the Presence of Ascorbic Acid in Wheat Leaves and the Consequences of Its Presence for Sugar Determinations

Before proceeding to discuss the interference due to ascorbic acid and its degradation products in reducing sugar determinations it will be advisable to consider some of the pertinent properties of this substance and its degradation products. Ascorbic acid is soluble in 80% alcohol. It reduces Fehling's solution in the cold and produces what is now known as dehydroascorbic acid (42). The dehydroascorbic acid produced will reduce Fehling's solution if the mixture is boiled (42). On being heated with 12% hydrochloric acid

* *Personal communication.*

it yields furfuraldehyde (19) and consequently will give the orcinol and phloroglucinol tests for pentoses. The first oxidation product, dehydroascorbic acid, is a neutral substance. Its alkaline solutions are yellow and very unstable. They show absorption bands at 340 mμ and 265 mμ which shift to 300 mμ and 245 mμ upon acidification (19).

Ascorbic acid is not precipitated from aqueous solutions by neutral lead acetate but it is precipitated by basic lead acetate (42).

Ascorbic acid is usually determined in biological materials by the use of the dye 2,6-dichlorophenolindophenol in the pH range 1.0 to 3.5. The effects of other interfering substances can be largely overcome by extrapolation of the progress curve of dye decolorization with time when colorimetric methods are used (22). The first oxidation product of ascorbic acid is determined in the same way after a pretreatment with hydrogen sulphide. According to King several biologically important substances interfere considerably with this determination (22). In the following experiments, the method of Morell (26) was used except that the determinations were carried out at 520 mμ on a Coleman Junior Spectrophotometer.

Ascorbic acid has been isolated from several plant materials as noted in the introduction. In orange peel a large fraction of the reducing power has been shown to be due to dehydroascorbic acid or diketogulonic acid (16). If we accept the decolorization of the dye as proof of the presence of ascorbic acid in a given plant material then a survey of Chemical Abstracts shows that, in plant materials, this substance is very widely distributed in appreciable quantities.

When wheat leaves are analyzed by the method of Morell for ascorbic acid, considerable quantities of this substance are found. In some cases alcoholic extracts of the leaves give positive tests with orcinol and phloroglucinol. Alcoholic extracts regularly contain small quantities of dehydroascorbic acid, as judged by the decolorization of the dye after pretreatment with hydrogen sulphide. These extracts are invariably yellow when alkaline, and very pale yellow or colorless when acid. They clearly show the presence of an indicator which changes its color at a pH slightly on the acid side of litmus. This indicator is thought to be the yellow degradation product of ascorbic acid rather than a phenolic substance since it is not soluble in ether and when Anderson (1) tried to hydrolyze the yellow ether insoluble material which he suspected to be a flavone glucoside he recovered no flavone. The alkaline extracts show a pronounced absorption band at 340 mμ and this band becomes much less intense in acid solutions. These results agree with the properties of dehydroascorbic acid solutions, as reported by Herbert *et al.* (19). The yellow material considered to be one of the degradation products of ascorbic acid is not to be confused with the ether soluble flavones which are present in Khapli wheat leaves and have been identified by Anderson and Perkin (1, 2).

Ascorbic acid and dehydroascorbic acid both interfere with the colorimetric resorcinol method. The absorption spectra produced by these two substances

when treated with the resorcinol reagent is given in Fig. 3. This figure shows the striking similarity between the absorption spectra produced by dehydroascorbic acid and wheat leaf extract under the conditions of analysis. It is

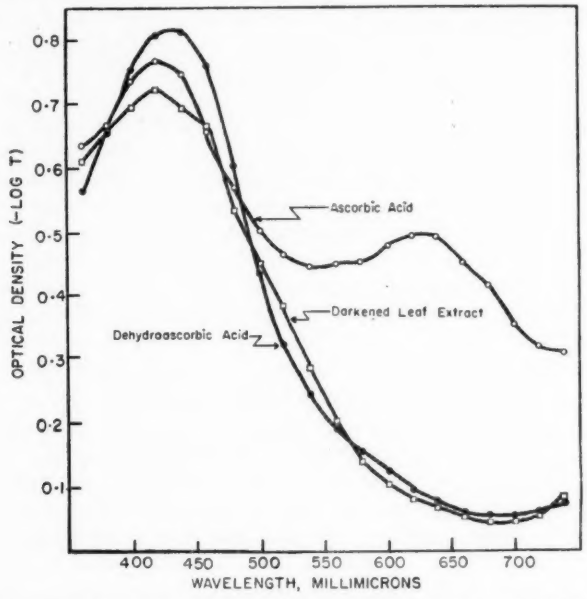

FIG. 3. *The absorption spectra of darkened wheat leaf extract, ascorbic acid, and dehydroascorbic acid after treatment with resorcinol and hydrochloric acid.*

therefore probable that dehydroascorbic acid or diketogulonic acid is an important interfering substance in this method of determining fructose residues.

In view of the several manifestations of the presence of ascorbic acid degradation products mentioned above an attempt was made to estimate the magnitude of the interference in the reducing sugar determinations from these sources. Since ascorbic acid is oxidized to form several substances, some of which have not been definitely identified (e.g. the yellow material), and since the extracts contain a mixture of unknown amounts of these degradation products, it is only possible to form a rough idea of the magnitude of interference that ascorbic acid produces in reducing sugar determinations. However the figures presented below show that ascorbic acid degradation products should be considered in interpreting reducing power determinations on plant material.

The first step in examining the interference of ascorbic acid degradation products was to study the reducing power of ascorbic acid, dehydroascorbic acid, and aged neutralized solutions of dehydroascorbic acid in known concentrations. The ascorbic acid solution used was standardized by titration

with 0.01 *N* iodine solution in potassium iodide. The concentration of dehydroascorbic acid in the solution was determined by the decolorization of the dye after treatment with hydrogen sulphide. The dehydroascorbic acid solution was produced by treatment of a portion of the ascorbic acid solution with activated charcoal and filtering. The dehydroascorbic acid solution produced in this way did not absorb any iodine and so was not contaminated with ascorbic acid. This solution had a pH of 2.5 and 70 ml. of it required 10 ml. of ca. 0.1 *N* sodium hydroxide to bring it to pH 7.0. This neutralized solution after being allowed to stand five days at room temperature, not protected from atmospheric oxygen, is referred to as the aged neutralized dehydroascorbic acid solution. The reducing powers of these solutions as determined by the Hanes method and the Somogyi method are given in Table IV. This table shows that, when dehydroascorbic acid decomposes,

TABLE IV

REDUCING POWER OF ASCORBIC ACID AND ITS DEGRADATION PRODUCTS

Substance	Concentration, mgm. per 5 ml.	Thiosulphate titers, ml. 0.01 *N*	
		Hanes method	Somogyi method
Ascorbic acid	2.51	6.66	9.79
Dehydroascorbic acid	1.48	2.20	4.54
Dehydroascorbic acid—neutralized	1.48	2.40	4.43
Dehydroascorbic acid—neutralized and aged	1.48	3.42	1.28

the degradation products have a greater reducing power as determined by the Hanes method than does the acid itself whereas the opposite is true in the case of the Somogyi method. Erratic changes of this type make it very difficult to predict the reducing power to be expected from a given amount of ascorbic acid degradation products when there is no way to determine individually the quantities of the reducing substances responsible for these changes. It is probable that the reducing power of the degradation products is influenced by the age of the extract which controls its composition.

The ascorbic acid in 26.8 gm. of wheat plant tops two and one-half weeks old was determined by the method of Morell (26). A sister sample of leaves, 86.4 gm., was extracted for one hour with 500 ml. of boiling 80% alcohol. This extraction was repeated on the residue and the extracts combined, evaporated to dryness at 40° C. under reduced pressure, taken up in water, filtered, and made to 100 ml. Aliquots of this solution were analyzed after suitable dilution for ascorbic acid and dehydroascorbic acid. The reducing powers by the Hanes method and the Somogyi method were measured in this uncleared extract. A part of this extract was cleared with basic lead acetate and sodium carbonate and then its reducing power was measured by both methods and its dehydroascorbic acid content was determined. The results of these experiments are given in Table V.

TABLE V

REDUCING SUBSTANCES IN WHEAT LEAF EXTRACT

Type of extract	Ascorbic acid, mgm./100 gm. fresh weight	Dehydro-ascorbic acid, mgm./100 gm. fresh weight	Reducing substances as ml. 0.01 *N* thiosulphate per 1 gm. fresh wt.	
			Hanes	Somogyi
Metaphosphoric acid	130	—	—	—
Uncleared alcohol	0.2	1.2	10.5	7.7
Cleared alcohol	—	0.6	5.1	3.9
Calculated from ascorbic acid determinations			2.8	0.7

During the dehydroascorbic acid determinations on the wheat leaf extract, drift of the colorimeter readings was encountered. A similar state of affairs was found in the aged neutralized ascorbic acid solution but not in the fresh dehydroascorbic acid solution. These results suggest that at least one of the degradation products of ascorbic acid interferes with the determination of dehydroascorbic acid.

The results of the quantitative determinations on the wheat leaf extract given in Table V show that dehydroascorbic acid is only partly removed from the extracts by basic lead acetate, and that approximately 25% and 10% of the reducing power in the uncleared extracts, as determined by the Hanes and Somogyi methods respectively, can be accounted for as ascorbic acid degradation products, if we assume that these degradation products have the same reducing power as the corresponding decomposition products in the aged neutralized dehydroascorbic acid solution. This latter assumption is not justifiable but it is the only possible assumption at present available to determine roughly the order of magnitude of the interference of ascorbic acid degradation products in reducing sugar determinations. These results do not rule out the possibility that almost all the reducing power of alcoholic extracts of wheat leaves is due to ascorbic acid degradation products. Diketogulonic acid, a degradation product of ascorbic acid, has been shown to be one of the major reducing substances in the juice of orange peel (16). It seems more likely that there is at least a second nonsugar source of reducing power in cleared alcoholic wheat leaf extracts besides ascorbic acid and its degradation products. This substance is not a nitrogenous base precipitable by phosphotungstic acid. It is surprising that approximately half the reducing material determined by both the Hanes and Somogyi methods was precipitated by basic lead acetate. It is therefore possible that the precipitated reducing material has the same composition as that left in solution. No experiments have been performed to find out the extent to which ascorbic acid degradation products are precipitated by basic lead acetate. In plant extracts this precipitation is probably influenced greatly by the pH of the extract during the

precipitation. The situation would then be similar to that found by Van der Plank (43) for sugars in plant extracts.

The Alcohol Insoluble Labile Carbohydrates

Since fructosans have been isolated from the leaves of some grasses under certain conditions, the possibility of their presence in the leaves of young wheat plants has been investigated. Although mature wheat leaves do not give a reaction for starch with iodine, they contain amylases. An effort was therefore made to find evidence for the presence of a substrate for these amylases in the leaves. With this in view dextrin and starch determinations were made in spite of the negative results obtained with iodine.

Wheat plant tops, two and one-half weeks old, were thoroughly extracted with several changes of 80% alcohol. The residue was dried, ground until it passed through a 100 mesh screen, and then the fructosan, dextrin, and starch determinations were made following the directions given in Loomis and Schull (24) except as noted below.

The reducing power obtained by the Hanes method after the hydrolysis for fructosans was equivalent to a fructosan content of 0.1 mgm. per gm. fresh weight of leaves. In order to find out whether this reducing power was due to liberated fructose residues an attempt was made to produce glucosazone from the supposed fructose residues. No yield was obtained. It is probable that much of the reducing power reported above as fructosans is due to other substances and so the fructosan figure given should be regarded as a maximum possible for the sample tested. It is concluded on the basis of these experiments that fructosans are present only in traces or not at all in young wheat leaves grown under our experimental conditions.

In the cold water extract, reducing power equivalent to 0.3 mgm. dextrin per gm. fresh weight was found after hydrolysis. In an attempt to obtain glucosazone from this preparation, a substance, presumed to be a phenylhydrazine derivative of something in the extract, was obtained. This material was soluble in hot water, and after crystallization from hot water had a melting point of 175° C., and a crystalline form different from that of glucosazone. This material was not identified. No glucosazone was obtained. Consequently the naphthoresorcinol test, as described by Browne and Zerban (7), was applied to the hydrolyzed extract. In this test a violet colored benzene layer and some dark colored insoluble material between the benzene and acid layers was obtained. This indicated the presence of hexuronic acids and suggested the presence of pentoses in the hydrolyzate. It was concluded that most of the reducing power reported above as dextrin was due to hexuronic acid, and/or pentose, condensation products which had not been removed from the extract by the previous clearing with neutral lead acetate.

After the cold water extraction of the leaf residue, a hot water extract was prepared. Upon hydrolysis it yielded a little reducing material which was not further investigated.

The residue, after hot and cold water extractions, was tested for starch with wheat leaf amylase after suitable pretreatment. No starch or other substrate for the amylase was found. Similar negative results were obtained with the hot and cold water extracts when these preparations were treated with wheat leaf amylase at pH 5.6 to pH 5.8.

The results of the investigation of the alcohol insoluble labile carbohydrates of Khapli Emmer wheat leaves indicate that the leaves do not contain appreciable quantities of fructosans, dextrins, or starch at the two and one-half week old stage when grown in the greenhouse in midwinter. No substrate was found in the leaves for the amylase which has been demonstrated to be active in the leaves grown under similar conditions.

Quantitative Determination of the Carbohydrates

In the previous sections, it has been shown that sucrose is probably the only abundant labile carbohydrate present in young wheat leaves. In the present section the problem of its quantitative extraction and determination will be considered. Consideration will also be given to the effects of various clearing procedures on the reducing power of the extracts and the recovery of added sugar.

Extraction

The first step in the quantitative determination of sugars in wheat leaves is to extract the sugars quantitatively. Most authors extract them with boiling 80% alcohol which inactivates all enzymes and greatly retards the hydrolysis of sucrose during extraction. The use of extraction times of several hours is frequent. In the case of a 5 gm. sample of wheat leaves 200 ml. of boiling 80% alcohol was found to extract almost all the carbohydrates in 15 min. A second extraction with a similar quantity of hot alcohol yielded very little additional sugar. Two 30 min. extractions with boiling 80% alcohol (200 ml. for each 5 gm. leaves) were adopted as a satisfactory procedure. Precipitated chalk is often added to these extractions to neutralize organic acids extracted with the sugars. In the experiments on wheat leaves no chalk has been added, and no evidence of hydrolysis of sucrose in detectable quantities has been obtained.

Clearing the Extracts

Unfortunately alcoholic extracts of plant materials contain substances which interfere with the determination of sugars by reducing power methods. Unfortunately also the identity of most of these troublesome substances is not known. Trouble may arise from a number of causes. First, the substance may have reducing power of its own (e.g. dehydroascorbic acid and other oxidation products of ascorbic acid), or it may yield reducing power after hydrolysis, as in the case of glucosides. Second, the interfering substances may alter the reducing power of the sugar towards the reagent employed. Well buffered solutions are likely to give this trouble especially if not neutralized before analysis. Unfortunately the pH at which reduction occurs

affects the amount of copper reagent reduced (32) and the amount of ferricyanide reagent reduced (46, also cf. Table II). This source of error is much greater with the micromethods where equal volumes of reagent and unknown are used than it is in the macromethods where relatively much larger volumes of reagents are used. It is a good reason for having all micromethod reagents as well buffered as possible. However other substances such as chloride ion influence the reducing power of sugar towards both copper and ferricyanide according to Van der Plank (43). Phosphates appear to have similar effects according to Van der Plank (43) and Englis and Becker (14). Van der Plank objects to the use of hydrochloric acid for the inversion of sucrose on these grounds but since its concentration was kept constant in the unknowns and the controls used for the calibration of the method, its effects will be taken into consideration (ignoring the effect, if any, of the chloride unavoidably present in the leaves). It is possible that the use of hydrochloric acid for the hydrolysis of dilute sugar solutions (2.5 mgm. per ml. or less) is responsible for the poor results obtained under these conditions with the copper reagent. This type of interference is probably present in wheat leaf extracts, since attempts to recover small amounts of added sugar have given very unsatisfactory results by both methods employed.

From the above it can be seen that it is probably impossible to eliminate all the sources of trouble in determining sugars in plant extracts by reducing power methods. The macromethods should be used if the quantities of material available permit their use since they reduce to a minimum all these sources of trouble except that due to nonsugar reducing substances.

Saturated neutral lead acetate solution, basic lead acetate solution of sp. gr. 1.25, and charcoal have frequently been used for clearing plant extracts for sugar determinations. In addition to the first two of the above, tests have been carried out with phosphotungstic acid and ammonium sulphate in order to try to shed some light on the nature of the reducing substances in wheat leaves.

Acidified phosphotungstic acid solution, chilled to reduce sucrose hydrolysis, was found to remove none of the reducing material not precipitated by basic lead acetate.

Since making the preparations saturated with ammonium sulphate had been found to remove almost all the reducing power from wheat leaf juice preparations used in enzyme studies, this technique was tested on the alcohol extracts. In these experiments solid ammonium sulphate was added to the preparations in sufficient quantities to make the solution saturated, the mixture was well shaken, stored overnight in the refrigerator, and then the precipitate was centrifuged down. The reducing power of the resultant supernatant was tested by the Hanes method. A similar test was carried out on material with a mixture of equal parts of glucose and fructose added to it. Table VI gives the results of these tests. The data show that sugar is much more readily precipitated by ammonium sulphate than is the reducing material in

TABLE VI

PRECIPITATION OF REDUCING MATERIAL AND SUGAR WITH AMMONIUM SULPHATE

	Hanes method ml. 0.01 *N* thiosulphate used	
	Reducing material	Sugar
Original extract	3.32	10.28
Ammonium sulphate treated	1.64	0.82
Per cent recovery after treatment	51	8

wheat leaf extracts. Assuming that all the material precipitated by ammonium sulphate is sugar, then only about half of the original reducing power is due to sugar. This is a maximum value for sugar. The rest must be non-sugar reducing substances. This experiment does not rule out the possibility that none of the reducing power in the extracts is due to sugar.

Charcoal has not been tested. It has been found to require careful handling by Roe (36) for use with urine while Van der Plank (43) considers its use unsatisfactory. Archbold (3) did not find charcoal completely satisfactory for use in the determination of all the sugars in barley leaves.

Both saturated neutral lead acetate solution and basic lead acetate solution of sp. gr. 1.25 have been tested on wheat leaf extracts. The use of basic lead acetate solution has been criticized by Bryan (8) since it partially removes sucrose and fructose from pure solutions of these sugars. Van der Plank carefully investigated the effects of treating plant extracts with basic lead and found that, as the basic lead acetate is added, the extracts become more acid at first and only become alkaline towards the end of the precipitation. It seems that the extracts never became sufficiently alkaline to cause precipitation of any sugar except fructose, even when basic lead acetate was used for clearing. He considers the loss of a little fructose as unavoidable. In the case of pure sugar solutions however the solutions do become alkaline enough that there is danger of losing sugar. Excess basic lead acetate must be avoided since the precipitate dissolves in the excess and so the good effects of its use are nullified.

Various deleading reagents have been used in the past to remove the excess lead used for clearing. Such substances are sodium carbonate, sodium or potassium phosphate, and potassium oxalate. The use of sodium carbonate has been criticized because it usually causes loss of sugar. With wheat extracts it has been found that, after deleading with carbonate, treatment with hydrogen sulphide yields a black precipitate assumed to be lead sulphide. This means that carbonate does not remove all the lead. In spite of the aforementioned objections carbonate gives satisfactory results with wheat leaf extracts. It is probable that carbonate does not cause loss of sugar on account of the low pH of the preparations during deleading. A few extracts were

encountered in the routine analyses that were acid to litmus even after deleading with sodium carbonate. Because phosphate, sulphate, and oxalate individually do not remove all of both the lead and calcium, Cook and McAllep (10) suggested a mixture of phosphate and oxalate as the best deleading reagent available. This reagent has been tested.

No experimental consideration has been given to the problem of removing ascorbic acid and its degradation products from wheat leaf extracts. This problem will be difficult to handle. Ascorbic acid itself is not precipitated by neutral lead unless the pH rises to 7.5, a value which would remove reducing sugars and possibly sucrose. Basic lead acetate precipitates ascorbic acid (42) but will not remove dehydroascorbic acid completely from plant extracts. Charcoal would not be satisfactory since it converts ascorbic acid to dehydroascorbic acid but does not remove the latter. It seems to remove the yellow degradation product of ascorbic acid but does not completely remove its colorless precursor (extracts decolorized with charcoal are always found to turn yellow again on standing for a sufficient time). Allowance can not be made for ascorbic acid by determining it in the fresh material since the degradation products causing the trouble have unknown and probably undeterminable reducing power with the methods now available. It is very probable also that the reducing power of the ascorbic acid degradation products in the extracts changes with time, especially if the solutions are slightly alkaline. It is well to remember that some plant materials e.g. walnut rinds, rose hips, paprika, currants, *Asclepias*, and many conifer needles have ascorbic acid contents in excess of 0.2% fresh weight, and that the substance is present in appreciable quantities in most plant materials, especially leaves.

Tests on Clearing Agents for Wheat Leaf Extracts

In the present set of experiments, tests were made on the extracts, and on extracts plus added sugar.

The effect of bisulphite as a possible clearing reagent was tested as it was thought that the interfering substances might be aldehydes or ketones that could be removed by this reagent. The results given in Tables VII and VIII show that this is not so. This does not rule out the possibility that the unidentified reducing substance postulated earlier is an aldehyde or ketone. In fact the behavior of the extracts towards 2,4-dinitrophenylhydrazine, after fermentation with yeast, suggests that there may be more than one aldehyde or ketone present in the preparation.

The solids from 300 ml. of the same extract as was used for the determination of the glucose and fructose residues were taken up in 50 ml. of distilled water. Four ml. samples were taken and cleared by the methods outlined below. Solutions of glucose and sucrose of known strengths were added in known quantities to some of the samples of the extract before clearing. In the clearing procedures, each reagent was added a little at a time, until it had caused maximum precipitation. The use of an excess was avoided in all cases. The precipitates were removed by centrifuging, washed once, and the washings

TABLE VII

SUGAR RECOVERY FROM WHEAT LEAF EXTRACTS USING THE METHOD OF SOMOGYI

Treatment	Clearing reagents*	Extract sugar, mgm.	Added sugar recovered, mgm.	Recovery, %
None			213	100
	a	117	220	103
	b	105	218	102
	c	108	207	97
	d	83	217	102
Invertase hydrolysis			1000	100
	a	175	975	97.5
	b	165	985	98.5
	c	185	915	91.5
	d	185	955	95.5
Acid hydrolysis			1061	100
	a	300	700	67
	b	260	1160	109
	c	375	875	83
	d	240	1135	107

* *See Table VIII for clearing reagents used.*

TABLE VIII

SUGAR RECOVERY FROM WHEAT LEAF EXTRACTS USING THE METHOD OF HANES

Treatment	Clearing reagents*	Extract sugar, mgm.	Added sugar recovered, mgm.	Recovery, %
None			242	100
	a	227	219	91
	b	207	248	103
	c	223	238	99
	d	172	232	97
Invertase hydrolysis			971	100
	a	178	975	100
	b	148	1047	107
	d	138	995	103
Acid hydrolysis			970	100
	a	163	1206	125
	b	183	927	96
	c	207	1027	111
	d	163	1243	128

* *Clearing reagents used:*

a—Saturated neutral lead acetate and sodium carbonate.

b—Saturated neutral lead acetate, saturated sodium bisulphite, neutral lead acetate, and sodium carbonate.

c—Saturated neutral lead acetate, and a solution of 7 gm. disodium hydrogen phosphate and 3 gm. potassium oxalate in 50 ml. water.

d—Basic lead acetate solution sp. gr. 1.25, solid sodium carbonate.

added to the supernatant. After clearing, the pH was adjusted to 7.0, prior to analysis by the Hanes and Somogyi methods described above. The clearing and deleading reagents tested are given in Table VIII.

The data in Tables VII and VIII show that there is very little to choose between the four clearing methods used as far as sugar recovery goes. The use of basic lead acetate gives slightly lower values for reducing sugar in the extract than does neutral lead acetate. Since it does not remove glucose, these lower values are undoubtedly nearer to the true values than those obtained using neutral lead acetate solutions for clearing. This result is in accordance with the view that most of the reducing power in these extracts is due to nonsugars. On this basis, basic lead acetate was chosen as the clearing reagent for the routine analyses. The poor recoveries of nonreducing sugar, after acid hydrolysis, are due to the low titration values in the case of the copper method. The behavior of the copper method is rather erratic with low concentrations of sucrose which have been subjected to acid hydrolysis, but this is not true with invertase hydrolysis. This erratic region extends up to sucrose concentrations of 0.5 mgm. per 5 ml. of sample analyzed. This was the only objection to the copper method that was encountered. Although the Hanes method gave a smooth calibration curve for low sucrose concentrations when acid hydrolysis was used, it did not yield good recovery values for sucrose from the extracts under these conditions. Invertase hydrolysis of the added sucrose was superior to acid hydrolysis in the recovery experiments regardless of the method of analysis used.

The experiments on added sugar and its recovery from wheat leaf extract do not establish one of the two tested methods of sugar determination as superior to the other. It is probably true that the values given for reducing sugar in the extracts by the copper method are nearer to the truth than those given by the ferricyanide method. A similar conclusion was reached by Heinze and Murneek (18) who compared several copper methods with the ferricyanide method of Hagedorn and Jensen. Both methods were used in the experiments on the gradients in wheat leaves to be reported later. An examination of the results (35) shows that where there is an appreciable quantity of sucrose present; the results of the methods of Hanes and Somogyi agree quite well as to its quantity. The fact that the results of these two methods agree so well with each other, and with those of the colorimetric resorcinol method (on the assumption that there is no free fructose in the leaves), leads to the conclusion that the values obtained for sucrose by these methods are quite close to the true value for the quantity of sugar present. If, however, true reducing sugars are present in the extracts being examined it would be better to use the copper micromethod of Somogyi or some modification of it as it seems to be much less sensitive to nonsugar reducing substances than is the Hanes method.

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AN ANALYSIS OF *CLADOPHLEBIS* (*KLUKIA*) *DUNKERI* SCHIMPER, A MESOZOIC FERN FROM WESTERN CANADA[1]

BY NORMAN W. RADFORTH[2] AND ARTHUR B. WOODS[3]

Abstract

The fossilized remains of frond fragments, occurring as carbonaceous compressions, have been investigated by the transfer method and other palaeobotanical techniques. The structural details revealed, particularly those related to morphological features of the fructifications, facilitate discussion on the phylogeny of the plant. Spores and spore characters disclosed for the first time have also proved important from phylogenetic aspects. The plant is more closely related to the carboniferous representatives of Schizaeaceae than to the more recent members of this family.

The carboniferous members of the family of Schizaeaceous ferns are represented by three species among which *Senftenbergia Sturi* Sterz. pro var., belonging to the Lower Carboniferous of Britain, is the oldest (Radforth (5)). The most completely described Mesozoic example of the family is *Anemia fremonti*, belonging to the Upper Cretaceous in Wyoming, U.S.A. (Andrews (1)). Prior to the time when detailed analyses of the morphology and taxonomy of the Carboniferous types had been reported (Radforth (4)) all certain evidence of the history of Schizaeaceae rested on the description of material of the genus *Klukia*, the infertile examples of which were assigned to *Cladophlebis*, a form genus designated on the basis of foliar morphology (Seward (6), p. 343).

The descriptive data relating to the morphology of *Klukia* fructifications are meager as compared with the information available for the fructifications of other genera of the family. This doubtless is due to the fact that the *Klukia* remains had escaped the critical analytical techniques applied by modern palaeobotanists.

Taxonomic and phylogenetic relationships embracing the earlier and the more recent members of this family of ferns have become manifest through the application of these new techniques. It has been the opinion of the authors that if *Klukia*, intermediate in age, were to be likewise examined, new information of a detailed morphological nature might be furnished which would contribute to a more complete and satisfying description of the fossil. Moreover, these new data would provide a demonstration of another significant link now required for a more complete elucidation of the taxonomic and phylogenetic implications.

The first major difficulty associated with the investigation was that of procuring specimens for analysis. The only material finally available was

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obtained from the Geological Survey of Canada collection in Ottawa, through the consent and kind assistance of Dr. W. A. Bell, Chief, Palaeontological Section, Geological Survey of Canada. The specimen used in the analyses carries the number 5385, and is from "locality 4035, upper part of York Creek, 200 ft. above the base of the Blairmore formation", lying in the Lower Cretaceous.

A second difficulty was experienced through the fact that the specimen presented very scant carbonaceous remains. It is now generally known that the majority of fossil plants exist either as simple impressions in the rock matrix or as carbonaceous compressions. Of these only the latter lend themselves readily to analytical treatment. In specimen number 5385 the fossilization was chiefly of the impression type, which accounted for the lack of carbonaceous remnants.

Limitations of Transfer and Maceration Techniques

Normally in applying the transfer technique to fossil compressions a microscope mount is ultimately obtained in which the undamaged surface of the carbonaceous material is freed from the rock matrix in which it was imbedded and made available for examination on a cellulose acetate film (Radforth (4)). Thus from one piece of rock one transfer is procured. If the amount of organic matter on the rock surface is slight the transfer derived will show little evidence of fossil remains. In applying the transfer technique it is also normally the case that any carbonaceous remains which are released with the rock matrix by the application of hydrofluoric acid are seldom retrieved for study if the amount of carbonaceous material on the ultimate transfer mount is adequate for the investigation. Because the latter did not hold in this study, care had to be taken to conserve all carbonaceous fragments belonging to the plant as they were exposed by the hydrofluoric acid.

To ensure against loss of material through accident, the rock was divided into portions. Hence, if the analysis failed for one fragment all hope of procuring the information required was not lost. In some instances fragments of the specimen which had probably been freed in the course of fossilization could be freed by local treatment with *aqua regia*, and these provided a secondary source of supply.

The transfer technique, an invaluable aid in this investigation, has been described elsewhere (Radforth (4), p. 385) and no further reference to it is required here.

Because the amount of carbonaceous material was so meager there was little opportunity to apply methods to encourage semitransparency of the coaly remains. Attempts on a limited scale were made, however, to investigate isolated fragments for this purpose. Shultze's reagent was utilized and employed as indicated in an earlier investigation (Radforth (4), p. 386).

The majority of the analyses have been directly dependent upon the use of the Leitz Ultropak, which permits the investigator to view the unmodified

carbonaceous matter under very critical optical conditions by reflected light. The use of this equipment as a palaeobotanical tool cannot be overemphasized. Without it, microscopic morphological details in many cases would go unnoticed, and where features which might be revealed with ordinary oblique incident light would be problematical, the Ultropak equipment brings out the evidence with the desired clarity.

In recording observations photographically, the most significant problem which arose was due to lack of contrast between the fragmentary compression and the very dark shale in which it was embedded. A similar difficulty was encountered with respect to photographic records of transfers owing in part to an insoluble deposit left on the transfer mount following the hydrofluoric acid treatment.

Description of Morphological Details Revealed in Analyses

Little information concerning structure of vegetative features can be ascertained from simple inspection of the exposed fossil surface on the rock. Examination of Fig. 1 will bear this out. Pinnule contours shown at *p* are difficult to discern regardless of lighting conditions, first, because of lack in contrast between the fossil and the rock matrix in the actual specimen, and secondly, the number of whole pinnules represented by organic matter was small, making the evidence fragmentary so far as pinnule form is concerned. In some instances where the adaxial pinnule surface was worn away or damaged, impressions or fragmentary remains of fructifications could be discerned with the aid of a hand lens. The irregular black patches at *C*, Fig. 1, mark the positions of some of these masses.

By dissecting away chips of shale, small pieces of pinnae were revealed which showed the characteristic cladophleboid form referred to in the general accounts in the literature, e.g., Seward (6), p. 348.

Apart from the slight obscuring effect of the insoluble mineral film covering the mounts, transfers were invaluable in supplying the best and most significant descriptive data from the analysis.

In the photograph showing a pinnule in abaxial view (Fig. 2) the pinnule margin is seen at *m* and as the black of demarcation suggests, the margins are slightly incurved.

The arrangement of the fructifications (Fig. 2) is difficult to determine. Apparently in fossilization they have been compressed into the pinnule surface at various angles and for the most part their points of insertion on the lamina are obscured. Only two rows of sporangia are present, however, one row on either side of the pinnule.

In the transfers as well as in the macerated shale the sporangia stand out in relief. They are ovoid in shape and apparently sessile (Figs. 3, 4). The frequency with which the sporangia are disclosed showing compression having occurred at right angles to their long axes is low. Thus a typical lateral view of a sporangium is not common. On this account it was difficult to procure

examples from which full-length measurements could be taken. Also, even where foreshortening of sporangia was apparently negligible, it became a matter of comparing various examples in judging which of those examined were mature.

The authors have agreed that full-grown sporangia attain a length of 0.38 mm. This is based on inspection and measurement. The width of a mature sporangium is much easier to ascertain because, though it may be foreshortened, the axes at right angles to the compression thrust are not reduced in fossilization. The regularly obtained measurement of 0.24 mm. therefore may be said to represent any diameter of the sporangium in the plane limited by its greatest circumference. The fructification shown in Fig. 5 approximates the ideal example from which dimensions may be procured. However, even this specimen is possibly slightly foreshortened owing to the angle in which it is preserved. The plane of the photographic film, which is horizontal, catches only a portion of the image in focus.

Reference to Fig. 5 will reveal an important structural detail characteristic of the sporangia—an apical zone in which the typical coriaceous appearance of the sporangial walls is greatly emphasized. This increased "wrinkledness" is not evident in every case, e.g., Fig. 3. On the other hand, in many cases (the majority) it is quite marked and becomes readily discernible when viewed with the aid of the Ultropak (Fig. 6). It frequently obtains that this zone is all that there is preserved of the fructification (Fig. 7). There can be little doubt that it represents the remains of the apical annulus of heavy walled cells.

Interrupting the annulus is the strip of thinner walled cells, which provides for dehiscence. This feature is perhaps best illustrated in Fig. 8, *d*. The dehiscence zone is from 7 to 10 cells wide and clearly defined. This feature is well displayed in the vertical view of a decapitated sporangium, Fig. 9. Here the zone of dehiscence lies between the two designations, *d*, *d*.

When a sporangium is viewed laterally, it is clear that the dehiscence zone almost connects the two extremities of the fructification. At the free apex it invades an apical patch of thinner-walled cells. In this respect its apical limit goes beyond that of the annulus. The latter is for the most part one large cell in depth, though there is some evidence suggesting that the annulus-type cells with the vertical, much thickened walls occasionally appear in a second as well as a typical first row which bordered the apical patch. This extension of the main part of the annulus leaves in some cases an indefinite proximal limit. This may be observed by inspection of the illustrations which give an apical view of the sporangium, Figs. 7, 9, 10, 11. The feature is perhaps less clearly shown in lateral views, but some evidence of proximal extension of the annulus is suggested by intervention of cross walls in Figs. 5, 7, 8, *c.w.*

It is difficult to determine the cell number comprising the apical patch of thin-walled cells. It is more difficult to photograph the condition. Some of the irregularities seen in the apical patch area, Figs. 7, 10, 11, *p*, may be

indicative of wrinkling in a cell wall at right angles to the line of vision rather than to ridges marking the edges of vertical walls. The collective evidence strongly suggests, however, that the number of cells exceeds one, but is not greater than three or four. Fig. 9 cannot be utilized to interpret apical patch structure because the distal end of the sporangium has been removed here and the contents, organic and mineral matter, are revealed.

Because of the scarcity of material, examination of sporangial contents has thus far been sharply limited. Analyses have been pressed far enough, however, to reveal the first evidence of spores. The tip of a single spore mass with remnants of sporangium wall attached is shown in Fig. 12. The opacity of the mass prevents critical examination of the contents. However, at the fringe two spores are discernible. One of these is over half exposed to view and affords some opportunity for examination. The mature spores approximate to spheroids though measurements do suggest axes of slightly unequal length. It is felt that the presence of one slightly shorter axis is related to the orientation of the spore in the tetrad. The diameter of the mature spore ranges between 30 and 40 giving an average figure of 35 μ. Parallel ribbing is clearly evident as a spore pattern feature. This character may also be observed in the immature spore, Fig. 13.

From the data procured relative to spore and sporangial size, it is possible to estimate to a reasonable figure the number of spores produced by a sporangium. In order to derive this, the volume of a mature sporangium or spore mass, and a mature spore must be calculated. The former prerequisite has

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All figures are from untouched photographs.

Cladophlebis (*Klukia*) *dunkeri* Schimper

FIG. 1. *Specimen from which analyses have been made. Note poor contrast between organic matter and rock matrix, also limited supply of organic matter, p.*

FIG. 2. *Portion of transfer showing distal extremity of an inrolled pinnule margin m and orientation of fructifications. Note poor contrast due to mineral film precipitated on the mount.*

FIG. 3. *Submarginal row of sporangia showing arrangement.*

FIG. 4. *View of single sporangium showing narrowed region of attachment.*

FIG. 5. *Single sporangium in lateral view slightly foreshortened. Note cross walls in annulus cells, c.w.*

FIG. 6. *Apical segments of two sporangia showing marked coriaceous appearance when seen with Ultropak.*

FIG. 7. *Apical views of sporangia compressed in a direction parallel with their long axes. Note cross walls in annulus cells, c.w.*

FIG. 8. *Highly magnified lateral view of a sporangium apex showing annulus, and zone of dehiscence, d. Cross walls in annulus cells, c.w.*

FIG. 9. *Vertical view of a decapitated sporangium showing annulus cells in cross section and dehiscence zone between d, d.*

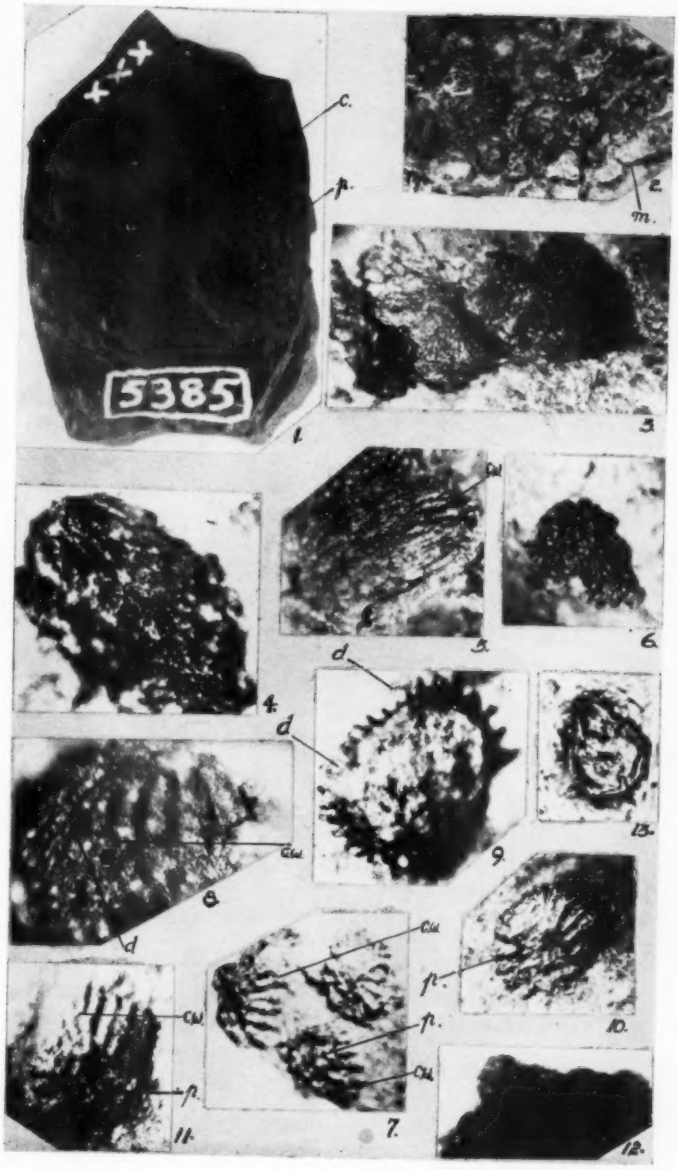
FIG. 10. *Vertical view of a complete sporangium showing apical patch distal to the annulus.*

FIG. 11. *Vertical view of a sporangium showing evidence of apical patch and cross walls c.w. in annulus cells.*

FIG. 12. *Lateral view of a mature spore at the edge of a sporangial mass. Note ridged pattern on spore.*

FIG. 13. *Isolated immature spore with abnormal projection on triradiate face. Note presence of ridges not very marked at this stage.*

PLATE I



been established as 0.012 cu. mm., the latter as 22×10^{-6} cu. mm. In calculating sporangial volume it is assumed that the sporangium approximates an ellipsoid with the semidiameter (short axis) as half the width of the widest part of the sporangium. Fructification volume divided by spore volume gives a value which must then be modified by applying the factor adjusting for the interspore spaces. This factor 0.74, has been referred to and utilized elsewhere (Radforth (5)) and a consideration of its derivation need not be given here. Once it is applied, the number of spores per sporangium works out to approximately 390.

Summary and Discussion

In spite of the lack of abundance of material the results of the analyses have clarified certain previously reported attributes and disclosed some new features. Chiefly the characteristics considered relate to fructification morphology, not to vegetative features. However, it is in the former category where information is required for taxonomic and phylogenetic purposes.

Photographic confirmation of the mode of attachment of the sporangia is not without significance. The early Schizaeaceous ferns possessed stalked sporangia. The suggestion has been presented that progressive reduction of the stalk in the course of geological time represents an evolutionary trend within the family. The disclosures made in this investigation support this view, since the *Klukia* (Early Cretaceous) type of attachment, along with the most specialized example of *Senftenbergia* (Upper Carboniferous), is sessile (or almost so) as it is in the related modern form, eg., *Anemia*. Also, from photographic evidence it would appear that the sporangial attachment for *Klukia* is at the proximal end of the fructification and not emerging at a point more distal as it is in the fructification of *Lygodium* with which sporangia of the fossil Schizaeaceae were formerly compared (Bower (3)).

Another morphological feature highly significant as a basis for comparison is the apical annulus. Depth of annulus in terms of rows of cells is a feature which may vary with species. This is the case with the Carboniferous species of *Senftenbergia* (Radforth (4, 5)), and to a lesser extent with the modern genus *Lygodium* (Bower (3), p. 169). However, among the Carboniferous examples a trend was apparent in which the most primitive type of sporangium in Lower Carboniferous times had the deepest annulus and the most advanced type the shallowest annulus (in terms of numbers of rows of cells). Also, the latter type represented in *Senftenbergia pennaeformis* was well defined and accompanied by a clearly marked zone of dehiscence in contrast to the former represented by *S. sturi* where both annulus and dehiscence zone merge gradually with the unspecialized tissue of the sporangial wall. Between the two extremes is the condition represented by *S. plumosa*. The evidence presented in this investigation suggests that the culmination of this trend was almost reached in early Cretaceous times. Typically, the annulus in the species of *Klukia* is only one cell in depth and the limits of the dehiscence zone are very distinct. The fact that occasionally the annulus depth approaches

the two-row type (*S. pennaeformis*) is perhaps to be expected. There can be no doubt, however, that it more closely resembles the single row type represented in the modern *Anemia*, where the trend culminates. It is instructive to note here, too, that this feature in *Klukia* suggests affinity with *Anemia* rather than with *Lygodium*.

Reference has been made in the section dealing with description to the apical patch of cells which is clear in the photographs. Though it is difficult to interpret the number of cells comprising the patch, there is good evidence supporting the view that the number certainly exceeds one, and probably two. It would appear that the shrinking of the apical patch (in terms of cell numbers) noted as a trend among the Carboniferous members of the family is carried little further in the species under investigation here. The condition is practically as it is represented in *Anemia*—a few cells—and contrasts with the condition in *Lygodium*, where the apical patch may be represented by a single cell.

A final consideration of sporangial structure should be made with reference to the contents. Bower's view, suggesting that in general spore output per sporangium is higher in the primitive living ferns than for the more advanced ones (Bower (2), pp. 262-3) was explored by one of the authors (Radforth (5)) with respect to the fossil and living Schizaeaceae. From this consideration Bower's view received support. The injection into the comparison on spore counts of 400 for this newly investigated member brings further support to the theory. The figure is far in excess of the spore output per sporangium for the modern species *Anemia* which is only 132.

Discovery in this work of the mature spore type for a species of *Klukia* in itself provides new information. The value of the discovery gains in significance when a comparison of the spore pattern with that of the other members of the family is made. Evidence disclosed in earlier accounts suggested a basic pattern of ridges and spines for the fundamental spore pattern exhibiting relationship among members of the family. Examination of the photographic evidence presented here suggests the member under investigation here is no exception to this, a point which appreciably supports the earlier reasoning.

A preliminary consideration of a comparison of vegetative attributes distributed among members of the Schizaeaceae indicates that *Cladophlebis* (*Klukia*) *dunkeri* Schimper more closely resembles the Carboniferous ancestors. Indeed what is known of its morphology contrasts with that of *Anemia fremonti* (Andrews (1)), a strikingly modern form, even though the two types belong to the same geological epoch. On the other hand, on the basis of reproductive aspects, the species of *Klukia* is well advanced comparing in some details favorably with *Anemia fremonti* and the modern members of this genus.

The complex might better be understood when investigations on vegetative structure accompanied by further study on reproductive aspects for other species of *Klukia* have advanced.

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